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Cultivation of Egg-Adapted Theiler's Mouse Encephalomyelitis (TO) Virus in Chick Tissue Culture. (20175)

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The present report describes the successful propagation of an egg-adapted strain of Theiler's mouse encephalomyelitis virus (TO) (1) in chick embryo tissue culture. The work was undertaken as part of an investigation of the problems involved in the cultivation of neurotropic viruses of low mouse infectivity. Poliomyelitis virus may be cultivated in human(2) and monkey(3,4) tissue. Maintenance in culture on tissues of other species has, however, presented difficulties. Similarly the TO type of Theiler's virus has not lent itself to cultivation, though the highly mouse-infective GD VII type may be readily grown(5).

Recent successful use of chick tissue for strains of Coxsackie virus which failed to grow on mouse tissue(6) prompted the investigation of the same medium for TO mouse encephalomyelitis virus.

Methods and materials. Viruses. The mouse encephalomyelitis (TO) strains were isolated in this laboratory from the intestines of albino mice(7). Stock strains have been maintained as infected mouse brain in 50% glycerol. The 40th mouse brain passage of strain No. 4727 was used to inoculate embryonated eggs. A suspension of infected embryo legs and wings from the 11th egg passage initiated the tissue

culture series. *Adaptation to eggs.* Fertile hens' eggs, incubated for 6 days at 37°C, were inoculated by the yolk sac route and reincubated for 10 days at approximately 35°C. The inoculation, harvesting of tissues, and preparation of suspensions were essentially as described for the Coxsackie viruses(6). Brains, legs and wings, and the abdominal and thoracic viscera were usually harvested. *Tissue cultures.* Suspended cell tissue medium in Erlenmeyer flasks, as used for the Coxsackie viruses, was used for the TO strains. Chick embryos, 6 to 8 days of age, furnished the tissue. Older embryos were less satisfactory. Usually the whole embryo, minus the eyes, was used; in certain experiments minced tissue from heads and legs was tested separately for its suitability as a substrate for viral growth. The first 14 passages were incubated for 10 days, subsequent passages for 7 days at 34°C. *Infectivity titers.* Mouse infectivity was demonstrated by inoculating 10- to 12-gram mice intracerebrally with 0.03 ml of the test material. To determine egg-infective titers, serial dilutions were inoculated into the yolk sac of 6-day embryonated eggs using as inoculum 0.1 ml of the chick embryo suspensions or 0.5 ml of tissue culture fluids. *Identity of strain.* The identity of the strain after egg or tissue cultivation was established by injection of the virus into 10- to 12-gram and infant mice, by histopathologic examination of the tissues of infected animals, and by challenge with the mouse passage strain No. 4727, with a representative strain of Theiler's virus (TO) obtained from the American Type Culture Collection and with the Lansing strain of poliomyelitis virus. For comparison the A.T.C.C. strain of Theiler's virus was also subjected to the above procedures for adaptation to eggs and tissue culture.

Results. Adaptation of virus to embryonated eggs. Strain No. 4727 adapted readily to growth in eggs. Virus was present throughout the embryo. A similar distribution of virus in the embryo has been reported by Riordan and Sá-Fleitas who carried a TO strain through 4 egg passages by inoculation on the chorioallantoic membrane(8). A suspension of the viscera of embryos of the second egg passage induced paralysis in 6 of 10 mice injected intracerebrally with 0.03 ml of a 10^{-4} dilution and in 1 of 10 injected with the 10^{-5} dilution. Subsequent passages were of similar titer, the brains containing less virus than the limbs and the viscera. Infective titers for eggs were approximately one log higher than for mice, possibly in part because of the larger inoculum used for eggs. The strain was transferred through 14 successive egg passages. In two passages some embryos were harvested 6 days, others 10 days, after inoculation; the longer incubation period gave a better yield of virus.

Infected embryos appeared to be stunted in comparison with uninoculated embryos of the same age but otherwise showed no signs of infection. Histopathologic examination revealed damage to the striated muscles but no lesions of the central nervous system. Mice inoculated with egg passage virus showed the typical signs of disease and lesions characteristic of the TO type of Theiler's virus(1). After 12 egg passages, the strain underwent a sharp drop in mouse virulence, which was not reversed by subsequent mouse brain passage. Two other strains were introduced into embryonated eggs. Strain No. 4771 grew readily, No. 47218 failed to adapt though repeated attempts were made. Both have approximately the same mouse infective titer. The A.T.C.C. strain of Theiler's virus also adapted readily to eggs.

Tissue cultures. Strain No. 4727 has been carried through 42 passages, the first 14 transferred at intervals of 10 days, subsequent passages after 7-day incubation periods. The infectivity titer of the 10th passage in tissue culture was determined both in mice and in eggs. Seven of 9 mice became paralyzed after intracerebral injection with 0.03 ml of a 10^{-1} dilution of culture fluid, 2 of 9 when a 10^{-2} dilution was used. Virus was present in quantity in chick embryos harvested from eggs inoculated with 0.5 ml of a 10^{-3} dilution, indicating the usefulness of the embryonated egg for detection of virus present only in minimal quantity. With *subsequent passages* increase in mouse-infective activity was suggested by the fact that the first paralyzed mice were occasionally noted 7 to 8 instead of 10 to 12 days after inoculation. The 31st passage was

TABLE I. Challenge of Mice Previously Injected with Egg-Adapted Theiler's Virus No. 4727.

	Challenge virus*			
	Theiler's virus No. 52102†		Lansing poliomyelitis virus No. 4701	
Preliminary inj.	10^{-2}	10^{-4}	10^{-1}	10^{-1} + anti-Lansing mouse serum
Controls				
None	8/8	5/8	7/8	0/7
Test mice				
No. 4727, gen. E-13				
2×10^{-1}	0/4		4/4	
10^{-1}	0/4		4/4	
10^{-3}	1/3		2/4	
10^{-5}	3/4		4/4	

* Challenge viruses were suspensions of infected mouse brains and cords. An interval of 5 wk elapsed between preliminary and challenge doses.

† American Type Culture Collection strain TO virus.

Denominator = No. of mice injected. Numerator = No. of mice paralyzed.

The previously injected mice weighed 25-28 g at time of challenges; mice of similar wt were used as controls. Mice were not paralyzed prior to inj. with challenge viruses.

TABLE II.

Challenge of Mice Previously Injected with Theiler's Virus No. 4727, Tissue Culture Strain.

			Challenge virus*			
	Survivors of 1st inj.		Theiler's virus, No. 52102			
Preliminary inj.	P†	NP‡	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Controls						
Tissue culture medium		18		5/6	6/6	3/6
10% normal mouse brain		18		5/6	4/6	2/6
Test mice						
Culture fluid No. 4727,		12	3/6	3/6		
40th tissue culture passage	11		0/4	0/7		

* The challenge virus was a suspension of infected mouse brains and cords. Interval of 5 wk elapsed between preliminary and challenge doses.

† Paralyzed in one or more legs. Suitable markings were used to identify paralyzed leg before injecting with challenge virus.

‡ Not paralyzed.

titrated both for mouse and egg infectivity. The highest dilution infective for mice was found to be 10^{-2} as before. On the other hand, virus was present in quantity in chick embryos inoculated with a 10^{-4} dilution of the tissue culture. Higher dilutions were not tested.

Minced tissue from legs of 8-day chick embryos, from heads or from the whole embryo served equally well as substrate for the propagation of the virus, differing from the findings of Pearson for the GD VII type which failed to grow in chick embryo body tissues other than brain and spinal cord(9). The *egg passage virus* was again adapted to tissue culture in a second series of transfers. On the other hand, success was never attained with mouse-brain virus similarly transferred to chick tissue culture. The A.T.C.C. strain of Theiler's virus also adapted to cultivation *in vitro,* using embryo tissue of the 1st egg passage as inoculum.

Identity of strain. The egg passage virus and the strain grown *in vitro* maintained the characteristics of the TO type of Theiler's encephalomyelitis virus. Infected mice developed flaccid paralysis after a relatively long incubation period (6 days or more) and otherwise showed little evidence of infection. The initial paralysis noted was usually in the hind legs. This localization of paralysis has characterized strain No. 4727 since its isolation, in contrast to other strains previously described(7). Infant mice showed no paralysis until 18 or more days following intracerebral

inoculation. Neither infant nor 10- to 12-g mice could be infected by the intraperitoneal route. *Histopathologic* examination of mice paralyzed after infection with tissue culture material revealed lesions similar to those induced by mouse passage virus.

Mice injected with egg or tissue culture virus were challenged 5 weeks later with Theiler's virus strains No. 4727, No. 52102 (A.T.C.C. strain), or the Lansing strain of poliomyelitis virus.

Discussion. With other viruses that we have investigated (MM, GD VII, Coxsackie) the titer of tissue cultures, even when well established, has been at least 2 logs lower than the mouse passage or egg passage virus used to initiate the series. In the case of strains of low titer such as Lansing poliomyelitis virus or the TO strains of mouse encephalomyelitis such a decrease may bring the viral content near or below the minimal infective level for mice. With strains which may be egg adapted this difficulty may be partially obviated by the inoculation of relatively large amounts of the cultures into embryonated eggs and subsequent testing in mice for the presence of virus.

The failure of one of 3 strains of similar mouse-infective titer to adapt either to egg or tissue culture suggests that strain differences exist within this group of viruses as well as among the Coxsackie viruses(6).

Strain No. 4727, *in vitro* or in the developing chick embryo, had no particular predilection for nervous tissue. In tissue cultures it grew on leg and skeletal tissue; in chick embryos infected via the yolk sac, lesions were found in the muscles and not in the central nervous system. This suggests that non-nervous tissue may offer a better source of essential metabolites for the multiplication of the virus than does brain tissue.

Summary. 1. An egg-adapted strain of Theiler's mouse encephalomyelitis virus(TO) has been carried through 42 passages *in vitro* on embryonic chick tissue. Attempts to adapt the mouse passage virus directly to chick tissue culture were unsuccessful. Preliminary growth in embryonated eggs, followed by tests in mice of 20% embryo suspensions, facilitated the titration of tissue culture fluids. 2. One of 3 strains of approximately the same mouse-infective titer failed to adapt either to egg or tissue culture. 3. A representative strain of Theiler's mouse encephalomyelitis (TO) virus obtained from the American Type Culture Collection also adapted to egg and tissue cultivation.

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Observations on Electrocorticographic Effects of Acetylcholine in Monkeys and Cats. (20176)

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(Introduced by W. H. Marshall.)

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Topical application of acetylcholine to the cerebral cortex of the cat and monkey has been reported to produce a local cortical depression, paroxysmal electrical discharges, and a spreading type of cortical depression(1-5,7, 8,12). We have repeated these experiments adding technics developed in this laboratory for the identification and control of spreading cortical depression of Leão(9-11). We have confirmed the local depressant and paroxysmal discharge effects of acetylcholine, but a spreading depression type of response was not obtained.

Method. Nine cats, anesthetized with nembutal 30 mg/kg or dial 30 mg/kg combined with nembutal 30 mg/kg, were used. One cerebral hemisphere was exposed and the electrocorticogram was recorded by means of 4 serially aligned wick electrodes usually placed on the suprasylvian gyrus. Each electrode recorded local D. C. potential (in mv range) in addition to brain wave activity(9). A single common reference electrode was placed on a saline moistened cotton pledget covered with mineral oil in contact with an area of exposed skull, this area was surrounded by a wall of dental plaster. In most cases arterial blood pressure was recorded from the femoral artery with a mercury manometer. Acetylcholine chloride (2% to 20%) was applied on filter paper pledgets approximately 4 mm square. The drug was also applied in droplet form from a No. 27 hypodermic needle to pledgets already in place. Care was taken to avoid mechanical stimulation of the cortex when these drugs were applied, and the cortex was maintained in a state as near physiological as possible for this type of experiment(10). The majority of trials were with 5 and 10% solutions. Similar experiments were done on 3 monkeys being used primarily for other experiments.

Results. Acetylcholine chloride produced several different electrocorticographic effects. These reactions were unpredictable and ranged from transitory local depression, through no discernible reaction, to a sustained convulsive type of activity. Repeated attempts to elicit propagated or remote effects by applying acetylcholine at finite distances from the recording electrodes met with failure. In contrast, application of the drug to pledgets monitored by an electrode failed to produce an electrocortical effect in only 3 of 48 trials (6%).

In accord with Forster and McCarter(5), it was noted that acetylcholine often induced a transitory diminution of activity followed by signs of hyperactivity. However, in contrast to the findings of Forster(3) and of Forster, Borkowski and McCarter(4), the temporary diminution in brain wave amplitude was local and static rather than migratory. In addition, the local depressant action was not associated with a significant or consistent D. C. voltage variation of magnitudes comparable with those observed in spreading depression of Leão. There was considerable scatter in the patterns of reaction. Isolated amplitude reduction with no subsequent evidence of increased activity occurred in 31% of trials. Approximately 19% of pledget applications resulted in local amplitude increase with no antecedent depression. In 44% of trials there was an immediate amplitude reduction followed within 5 to 10 minutes by increased electrical activity. Thus approximately 63% of trials showed signs of hyperactivity. Others have reported excitatory reactions in 58% of trials. The addition of Locke-Ringer solution to a pledget previously interposed between electrode and cortex failed to produce any of the electrical changes described above. Shunting, therefore, would not explain the temporary reduction in electrical activity.

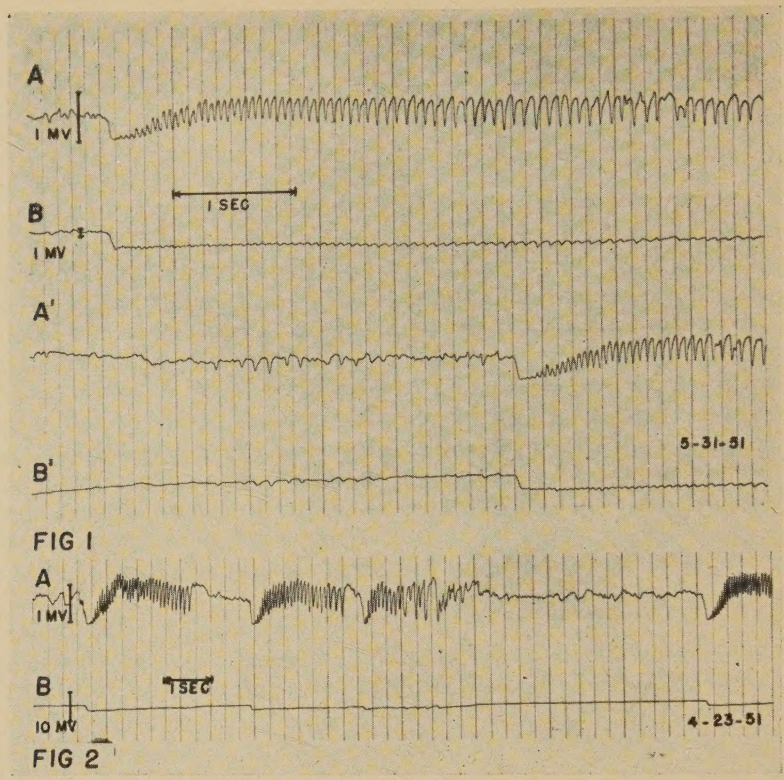

FIG. 1. Example of pulse-burst activity evoked by topical application of 10% acetylcholine to cortex of cat. A and B are records from the same electrode: Record A—through reactive coupled amplifier at sensitivity shown; record B—through direct coupled amplifier at approximately 0.1 sensitivity of record A. A′ and B′ are continuous with A and B reading time left to right. Record A-B has been cut and the contiguous section A′-B′ is mounted below it. This sample of record taken from dorsal striate 10 min. after application of 10% acetylcholine to area around electrode. Positive deflection is up in all records.

FIG. 2. A second example of this type of acetylcholine reaction from another cat. This record is from the mid-dorsal suprasylvian gyrus 16 min. after topical application of 10% acetylcholine.

In about 25% of 60 applications of acetylcholine the high amplitude activity was organized into brief bursts, each sequence of which was accompanied by a D. C. base line shift of 0.5 to 3.0 mv in the surface negative direction (Fig. 1). This burst sequence can be triggered by strychnine spikes produced by application of strychnine to the same region.

Local application of acetylcholine to the cerebral cortex usually produces a transient fall in systemic blood pressure. Hoff *et al.* (6) have reported similar results with methacholine. In agreement with Forster *et al.*(4) we also found that the transient fall in blood pressure was not the cause of the transient diminution of brain wave activity.

The electrocortical alterations produced by local acetylcholine application were less well defined in the monkey, but were otherwise basically similar. All effects are purely local, and various forms of acetylcholine discharges were seen in about 38% of trials.

Discussion. The somewhat variable results obtained in these experiments warrant only limited discussion. In summary, acetylcholine produces a gamut of purely local electrocortical alterations when applied to the exposed cerebral cortex of anesthetized cats and monkeys. No variety of spreading depression type of reaction was obtained. The concurrent incidence of burst spiking and a D. C. voltage shift is an intriguing phenomenon. However, the variability of the D. C. shift amplitudes makes causal interpretation difficult. The long

duration of the acetylcholine effects indicates the complexity of the reaction since the acetylcholine must be rapidly split by the esterase in the cortical tissue.

Summary. 1. Topical application of acetylcholine usually produced a local depression. The depression was not propagated, and was not due to changes of systemic blood pressure. 2. The local depression was often succeeded and sometimes masked by enhanced electrical reactivity. This action varied from a barely discernible increase to a sustained high voltage pattern lasting 30 minutes. The discharges were randomly distributed in time in some instances; in others, the activity was organized in distinct groups of paroxysmal spikes each group being accompanied by a distinct surface negative D. C. pulse.

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Relationship of Native Heparin to Clearing of an Alimentary Lipemia.* (20177)

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The dramatic *in vivo* clearing by commercial heparin and heparin-like substances of an alimentary lipemia has been noted by a number of workers(1-5). This clearing has been shown to be reversible in dogs and rats by injections of protamine(6), a substance which is known to combine with and inactivate heparin(7). In the intact dog it has also been shown that the clearing produced by small amounts of intravenous heparin is not initiated in the circulation of the brain, but is initiated in the circulation of the leg and lung; once circulation is complete and mixing of the blood has occurred clearing continues in samples of blood from all of these sources(8).

To test the effects of native heparin on an alimentary lipemia, anaphylactic shock and shock following the injection of certain histamine liberators have been studied. In other experiments the effects of intravenous injections of dextran have been observed. These studies will now be reported.

Material and methods.† Dogs were sensitized with 50% eggwhite (1 ml/kg). The initial injection was given intravenously and 5 and 7 days later this dose was repeated subcutaneously. Thirty days later the dogs were fed cream fat (2-4 g/kg body weight), and 3-4 hours later anaesthesia with nembutal was induced. A control lipemic sample of blood was then drawn. The small shock dose of antigen was injected intravenously. Four

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† We are indebted to Miss A. Grimsgaard for technical assistance.

TABLE I. Effects of Circulatory Shock, Protamine and Dextran on Blood Heparin and Alimentary Lipemia in Dogs.

Procedure	Dose range	No. of exps.	Response: Blood heparin*	Response: Visible lipemia
Anaphylactic shock	Shock dose: 1–5 cc 50% eggwhite	2	I	D
		1	I	N
		2	I	I
		1	N	N
		2	N	I
Compound 48/80	0.4–1.0 mg/kg	1	I	D
		2	I	I
		3	N	D
		1	N	I
DA 10	0.6 and 1.0 mg/kg	1	I	D
		1	I	V
Protamine (following shock)	2–4 mg/kg	3	D	I
		3	N	I
		1	N	N
Protamine†	8 mg/kg	1	I	I
Histamine	5 and 10 μg/kg	2	N	N
Dextran		3	N	N
		3	N	S
		3	N	D

* Changes in blood heparin activity refer to changes from normal values except in the case of "protamine (following shock)" where a decrease from elevated values (due to the preceding shock) occurred in 3 exp.

† Protamine sufficient to produce shock was inj. in this exp.

I = increase; N = no change; D = decrease; V = variable; S = slow decrease.

blood samples were then obtained during the first 10 minutes, and 2 more in the subsequent 20 to 30 minutes. In a few instances the animals died in approximately 10 minutes. The blood samples were drawn from the femoral artery into syringes containing 1/10 volume of 0.1 M sodium citrate. Sufficient blood was also drawn into heparin for determination of the chylomicron counts. Blood pressure was recorded from the carotid artery in order to estimate the severity of the anaphylactic shock. Experiments were also performed on normal cream-fed dogs which received small shocking intravenous doses of the potent histamine liberators diamino decane (DA 10) and compound 48/80,‡ blood samples being drawn as indicated above. In these the blood pressure usually fell to 40 to 70 mm of mercury within ½ minute after the injection. In other experiments the effect of protamine sulfate on fat clearing due to shock was investigated. Finally the effects of dextran§ (molecular wt 75,000) upon a cream lipemia were observed. In most cases fat clearing was estimated by inspection for visible turbidity of the plasma and by whole blood chylomicron counts under dark field illumination. Blood heparin was determined by metachromatic assay, after precipitation of the plasma proteins with 80% phenol(9). Antithrombic potency of the extracts(10) and thrombin inactivating power of the serum(11) were also estimated in many experiments. Clotting times were performed in duplicate by the capillary tube method.

Results. The results of our experiments are summarized in Table I. In 4 experiments the release of native heparin during anaphylactic shock or shock due to injections of histamine liberators was accompanied by clearing of an alimentary lipemia. On other occasions, however, we observed that the presence of large amounts of heparin in the blood during anaphylactic shock (3 experiments) and after injections of 48/80 (2 experiments) was not accompanied by lipemia clearing. In one experiment both whole blood chylomicron counts and the plasma turbidity were still unchanged

‡ Compound 48/80 was kindly supplied by Dr. E. J. deBeer of Wellcome Research Laboratories, Tuckahoe, N. Y.

§ Furnished by Commercial Solvents Corp., Terre Haute, Ind.

from the lipemic levels 14 minutes after the injection of the shocking dose of eggwhite, despite the presence of 1.55 metachromatic units (Mu) of native heparin per ml of blood. In 4 other experiments the lipemia (turbidity and chylomicron counts) actually increased following the onset of shock although the blood heparin continued to rise during the same period. (In our experience the normal range of blood heparin activity in dogs is 0.10 ± 0.08 Mu/ml and the threshold for lipemia clearing by commercial heparin may be less than 0.01 Mu/ml above the control value)(8).

Total clearing of a heavy lipemia may occur in dogs in the complete absence of antithrombic activity, and with no detectable increases in the level of metachromatic activity in the blood extracts. Also no increase could be detected in the thrombin inactivating power of the serum. In 3 experiments this was observed after the intravenous injection of 8 cc of 15% dextran (M.W. 75,000) into lipemic dogs, in which moderate to marked clearing occurred within a few minutes.‖ It is to be noted that these animals received several injections of dextran at intervals of about 1 week, making it possible that they had become sensitized to the dextran. This could have been a factor only in one experiment in which the maximum clearing was noted. Clearing of the lipemia in the absence of a detectable increase in native blood heparin activity has also been observed after shock due to injections of compound 48/80.

Following the injection of protamine sulfate the lipemia which disappeared in the presence of heparin reappeared promptly in all of our experiments. Protamine sulfate also reversed the clearing effect of compound 48/80 in lipemic dogs, even when the clearing was accompanied by no demonstrable increase in heparin activity in the blood of the animals.

Discussion. The observation that the release of native heparin in the blood stream of dogs may be accompanied by rapid clearing of a heavy alimentary lipemia supports the view that heparin may play an important physiological role in fat transport and metabolism(8). However, in only 4 out of our 10 shock experiments involving a rise in blood heparin was there any finding to suggest that native heparin might be the primary cause of lipemia clearing. In 5 other experiments increases in blood heparin coincided with either no change or an actual rise in the visible lipemia of cream-fed dogs.

While injections of commercial heparin in minute amounts usually produce some clearing of a lipemia, we have observed that the presence of even *much greater* amounts of native heparin in the blood may not be accompanied by clearing. It is possible that the clearing may be inhibited by other substances released into the blood stream during shock, or as previously suggested(8) the mechanism of clearing may be exhausted by its too frequent or prolonged use. On the other hand in 6 experiments, marked clearing was observed in the absence of measurable increases in the levels of heparin in the blood.

Our observations do not support the statement that heparin is essential for the production of a lipemia clearing factor(12). Neither do they show that the presence of adequate amounts of heparin necessarily cause clearing. We have shown that the injection of extremely small amounts of heparin in dogs may result in transient slight lipemia clearing even when these doses are too small to become manifest as an increase in blood heparin concentration (8), an observation which suggests the possibility that the methods of heparin assay may not accurately disclose the effective amounts of heparin in the blood. However, the notable persistence of a lipemia in the presence of adequate amounts of native heparin and the very marked clearing sometimes observed in the absence of detectable amounts of heparin are difficult to explain. Possibly alternate pathways exist, independently of heparin, for lipemia clearing to occur.

Summary. Studies have been made to determine the effects of native heparin upon the lipemia of cream fed dogs. It was found that shock due to anaphylaxis or to the injection

‖ It has also been shown by one of us (R.L.S.) that dextran (N.W. 75000 and 210000) will clear a lipemia *in vitro* when added to heparinized blood in concentration of 1% and 2%. When added to lipemic plasma this clearing is either entirely absent or very much less marked.

of certain histamine liberators sometimes causes heparin to appear in the blood of the animals. The presence of native heparin in the blood, however, does not necessarily coincide with clearing of a lipemia; fat clearing may occur during shock either in the presence or in the absence of detectable amounts of native heparin in the blood of dogs. In several experiments, the injection of dextran (M.W. 75,000) produced marked clearing of a heavy alimentary lipemia in the absence of heparin activity in the blood of dogs. Protamine sulfate caused the return of both visible turbidity of the plasma and of whole blood chylomicron counts to control levels after fat clearing due to shock. This occurred both in the presence and absence of significant blood heparin activity.

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Comparative Study of Epinephrine and an Epinephrine-like Substance Obtained from Autolyzing Adrenal Glands.* (20178)

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During an assay for adrenalin from minced, autolyzing adrenal glands which have been placed in dialyzing bags it was noticed that the dialysate had acquired a bluish-violet color. The pressor substance could not be precipitated from the dialysate by the addition of strong ammonium hydroxide but yielded epinephrine on acid hydrolysis. When the glands were sliced with stainless steel knives the bluish-violet color was not obtained. Suspecting contamination from the food chopper, we added various metallic salts to a purified adrenalin solution and it was found that ferric ammonium oxalate gave a comparable color. Solvents such as ether, chloroform, benzene and carbon tetrachloride failed to extract the color. In comparing the bluish-violet dialysates with Parke Davis (P.D.)† adrenalin to which ferric ammonium oxalate had been added, 10% HCl was added to both solutions; the dialysate turned colorless and the solution of adrenalin and ferric ammonium oxalate became a light yellow color. When 10% NaOH was added to the adrenalin and ferric ammonium oxalate an orange precipitate formed, but when 10% NaOH was added to the dialysate it turned pink. Both solutions contained 0.05 mg of adrenalin per ml as shown by bio-assay. P.D. adrenalin was used as the standard.

Folin(1) has shown that uric acid and Doty (2) has shown that ascorbic acid, both contained in adrenal glands, can interfere with the formation of colored compounds of epinephrine and certain metallic salts. It was found that ascorbic acid prevents the formation of the bluish-violet color of ferric ammonium oxalate and adrenalin but that uric acid neither prevents the formation of the

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† Parke Davis adrenalin.

color nor does it give the color itself as it does in Folin's method. It seemed advisable to us that if this color formation was to be made the basis of a method for the determination of adrenalin, that ascorbic acid should be prevented from interfering. It was unknown to Folin in 1912 that adrenal glands contain ascorbic acid, but Szent Gyorgy(3) found in 1928 that adrenal glands contain 600 mg of ascorbic acid per kg. Ascorbic acid also affects the formation of color in Folin's method. Edlbacher and Leuthardt (4) state that ascorbic acid and minute amounts of copper will inactivate urease. We found that the reverse was also true and that if a solution of adrenalin and ascorbic acid is heated with 0.1 g of urease but without the addition of copper for about 10 minutes the ascorbic acid will not prevent the formation of color when ferric ammonium oxalate is added.

Since the color development of epinephrine and ferric ammonium oxalate is more stable and also more sensitive than the color developed by Folin's method or Doty's method and since uric acid does not interfere in our method and ascorbic acid can be blocked, we are giving the procedure to be followed for the determination of epinephrine in adrenalin filtrates or precipitates.

Method. To a 12.5 mg sample in 250 ml of H_2O, add 2 ml of a 10% solution of ferric ammonium oxalate ($Fe(NH_4)_3(C_2O_3)_3 \cdot 6H_2O$), (stable at least one week). Shake well and let stand for 10 to 15 minutes, then read in a spectrophotometer at 555 mμ (or wavelength which is the center of extinction for the standard used. (Fig. 1). A water blank should be used. When transmittancy is plotted on semilogarithmic paper, the curve is not exactly linear except at midportion, and the greatest accuracy is between 45% and 65% transmittancy. This method is accurate from less than 1% to 5% as the greatest variation, depending upon the concentration being used. If the concentration of epinephrine is very small and the green of the reagent masks the characteristic blue color, it is advisable to use a larger sample and less of the reagent. The accuracy of a Beckman D. U. spectrophotometer varies as much as 6% at 373 mμ when compared to

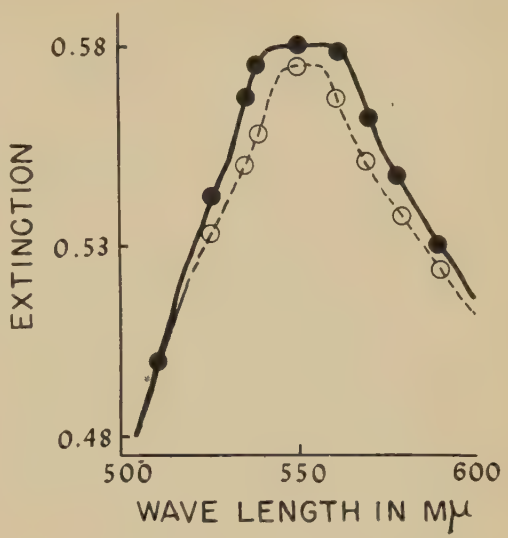

FIG. 1. Broken line represents extinction curve obtained by using P. D. (Parke Davis) adrenalin and ferric ammonium oxalate. Unbroken line represents extinction curve obtained from autolyzing adrenal glands. P. D. adrenalin curve is slightly sharper than the curve from the dialysate but both curves center at 552 mμ. Depth of the absorbing liquid was 1 cm and water blanks were used. The instrument was a Beckman model DU spectrophotometer. The P. D. adrenalin was a sample that had been in the laboratory for a considerable length of time. When a comparatively fresh (dated) sample of P. D. adrenalin was used the center of extinction fell at 575 mμ. Epinephrine samples from other sources were as follows: Armour & Co. U.S.P. suprarenalin, extra white, No. K47404 at 572 mμ; Armour & Co. U.S.P. epinephrine No. K47504 at 578 mμ; an epinephrine fresh sample prepared from adrenal glands by methods of Aldrich & Abel(8,9) at 585 mμ; Eastman No. 3097 at 572 mμ; Matheson No. 2835 at 575 mμ; and Levo-Arterenol Bitartrate · H_2O No. N-087-HD at 580 mμ. The center of extinction can vary by as much as 33 mμ. The data indicate that when one is using "so called" epinephrine standards, the manufacturer of the standard or the method of preparation that was used and also the shelf life of the standard should be kept in mind.

the average values of 11 other instruments, according to a U.S.P. collaborative assay(5). At 555 mμ the accuracy should be somewhat greater.

Uric acid does not interfere with the test but ascorbic acid prevents the formation of color. However, this can be prevented if 0.1 g of urease is added to the sample in about 25 cc of H_2O and heated (not boiled) for 10 minutes, then diluted to 250 ml and the reagent added. This solution can be filtered through

a fine sintered glass filter or cleared by adding $Al(OH)_3$, centrifuging and decanting.

Discussion. Apparently the ferric ammonium oxalate as such is not specific for color development since other ferric salts and oxalates together with adrenalin give similar results. Ferrous salts and oxalates when added to adrenalin give no color. Two compounds somewhat similar to epinephrine in structure, catechol and pyrogallic acid give a bluish color with ferric ammonium oxalate. However, they do not interfere with the quantitative determination. Other compounds such as tyrosine, quinhydrone, resorcinol, orcinol, phloroglucinol and ephedrine form yellow or pink colors and also do not interfere with the quantitative determination of epinephrine.

The bluish-violet color does not form in acid or basic solution outside of pH range 5.5 to 8.0; therefore the test is not applicable to blood since the usual methods use acid to precipitate the protein. In the method of Bloor and Bullen(6) for the determination of adrenalin in blood, the adrenalin reduces arseno molybdate to a colored compound. The method developed by Doty(2) was primarily for the determination of epinephrine in pharmaceutical products. In this method ferrous salts react with epinephrine in the presence of an alkaline buffer at a pH range of 8.0-8.5 to form a red-blue color having a maximum absorption at 530 mμ. A similar procedure was mentioned unfavorably by Barker, Eastland and Evers (7) who state that, "A blue color appears which is fairly stable but which fades rapidly to a purple color if too much alkali is added. The reaction did not seem sensitive enough for the estimation of traces of adrenalin." In the method of Doty(2) considerable attention has been paid to the colorimetric properties of certain compounds such as procain hydrochloride, thiourea and ascorbic acid which are often found in local anesthetic products. In regard to ascorbic acid Doty states that slight interference results from its presence in concentrations of 2 mg per ml providing the absorption is measured soon after the addition of the reagents. Among the stabilizers mentioned in this method and which have chromogenic effects, the only one which we have considered is ascorbic acid. Our method is intended for the estimation of epinephrine in adrenal glands only. In this respect it resembles the method of Folin, Cannon and Dennis(1), but with improvements in regard to handling ascorbic acid and uric acid and adaptation for use with spectrophotometers.

Summary. A bluish-violet color stable for 24 hours in a pH range of 5.5 to 8.0 results from addition of a solution of ferric ammonium oxalate to a solution of epinephrine or of suprarenal gland extracts. The transmittancy of the solution is determined at a wavelength of 555 mμ. The presence of ascorbic acid, uric acid or other naturally occurring substances found in adrenal glands does not interfere with the determination. This method is not applicable for the determination of epinephrine in blood or in drugs. The procedure is extremely simple and rapid and is capable of an accuracy of less than 1% to 5% as the greatest variation. The bluish-violet color that is obtained from minced autolyzing adrenal glands has been shown to be caused by a reaction between an epinephrine-like substance and iron products from the food chopper. A comparison of the specific extinction of various epinephrine standards and a note on the effects of dry storage is made (Fig. 1).

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In vivo Inhibition of Blood Agglutinins by Polysaccharides from Animal Parasites.* (20179)

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Polysaccharides were prepared from the larval form of *Trichinella spiralis* in rat muscle, and from adult *Ascaris lumbricoides* from pig. The well dried and pulverized worm material was suspended in water and placed in a boiling water bath for 30 minutes with constant stirring. The mixture was allowed to cool and then centrifuged for 15 minutes at 4000 r.p.m., the sediment being discarded. Sodium chloride was then added to the supernatant to make a 0.5% solution, followed by treatment with 4 volumes of 95% alcohol. The polysaccharide settled to the bottom after storage of the mixture at 6°C overnight. The supernatant was decanted, and the sediment centrifuged in 50 cc tubes and finally collected in one tube. Three washings, first in 95% alcohol, and 2 in ether then followed until the material remained dry. Partial purification of the polysaccharide was accomplished by resuspension in 0.5% sodium chloride, discarding the insoluble portion and reprecipitation with alcohol. This procedure was repeated at least 3 times. The final product was a clean, white powder, very soluble in water, giving a negative Biuret test for proteins.† Solutions of polysaccharides prepared from the trichina larvae and ascaris adults were injected intravenously into rabbits and monkeys. Also a commercial starch preparation from corn was injected as a control. The rabbits utilized were of both sexes and weighed from 3000 to 3500 g. Two female Rhesus monkeys were used, weighing 8 and 8.5 lb. The polysaccharide, dissolved in 0.85% sodium chloride, was injected intravenously into those animals whose serums had been previously found to have positive agglutinin titers at 37°C against human erythrocytes of Groups A_1 and A_2. The polysaccharides were also injected into animals with previously known negative titers at 37°C, so as to be able to study the effect of the polysaccharides on the agglutinins acting at 6°C. The animals were bled before, and 1, 6, 24, 48, and 96 hours after injection of the polysaccharides. The serums were always separated within 30 minutes after withdrawal of the blood. Before and after injection, serums were then tested for their content of agglutinins against human, sheep and the animals' own erythrocytes, following incubation at 37°C for one hour and after storage at 6°C overnight. The technic for the agglutination test has been described previously(1). The *human erythrocytes* utilized were obtained fresh from blood donors attending the blood bank of the School of Medicine. They were washed 3 times in 0.85% sodium chloride before testing. Erythrocytes from the same individual and from the same batch were used for the tests to be carried on for each experiment, which consisted in the testing for agglutinins within a 96-hour period. All erythrocytes were preserved at 6°C in Alsever's solution.

Results. Inhibitory effects of ascaris and trichina polysaccharides on blood agglutinins. Agglutinins at 37°C. The titers of the agglutinins in rabbit serums against human erythrocytes of Group A_2 were negative on the 1-, 6-, and 24-hour period after injection. Positive agglutination was observed, however, beginning at the 48-hour period, titer remaining lower than the one observed before inoculation. Similarly, the agglutinins in monkey serums against Group A_2 erythrocytes, were negative at the 1-, 6-, and 24-hour periods and remained low at the 48- and 96-hour periods. (See Table I). The *rabbit and monkey serums* gave positive titers against sheep erythrocytes before the inoculation of the polysaccharides, which became negative at the

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† The raw polysaccharide material from ascaris was sent by Dr. D. W. MacCorquodale, from Abbott Laboratories, Chicago, Ill.

TABLE I. Agglutinin Titers against Erythrocytes before and after Injection of Polysaccharides. Dose of ascaris, 500 mg.

Species wt, kg	Time rel. to inj.	37°C				6°C			
		A_1	A_2	Sheep cells	Own cells	A_1	A_2	Sheep cells	Own cells
Rabbit, 3.1*	Before	64	16	8	0	64	32	32	16
	+1 hr	2	0	0	0	4	0	4	4
	6	2	0	0	0	4	0	4	4
	24	8	0	0	0	16	8	8	8
	48	8	1	0	0	16	16	8	8
	96	16	2	0	0	16	16	8	8
Monkey, 3.6*	Before	256	32	8	0	4096	256	128	64
	+1 hr	16	0	0	0	256	16	8	8
	6	16	0	0	0	256	32	8	8
	24	16	0	0	0	512	64	16	16
	48	32	4	0	0	1024	64	32	32
	96	32	4	0	0	1024	64	32	32

* Results in additional rabbits and monkeys inj. with either ascaris or trichina polysaccharides were essentially the same.

1-, 6-, 24-, 48-, and 96-hour periods of examination. There was a decrease in titer of agglutinins against human erythrocytes of Group A_1, but these never became negative as observed with Group A_2 erythrocytes (see Table I). Starch did not affect the agglutinins at 37°C against any of the erythrocytes.

Agglutinins at 6°C. (Cold agglutinins). The agglutinins at 6°C against erythrocytes of all types were markedly reduced in the rabbit as well as in the monkey serums after inoculation with the ascaris and trichina polysaccharides. (See Table I). The diminution in titers was greater when a dose of 500 mg of polysaccharide was injected.

The serum from rabbits which had no agglutinins acting at 37°C, agglutinated the erythrocytes at 6°C. The polysaccharides from ascaris and trichina also reduced the cold agglutinin titers in these rabbits' serums.

Starch did not affect the agglutinins at 6°C against any of the erythrocytes.

Discussion. Polysaccharides from animal parasites have been previously reported to inhibit the isoagglutinins when added to human serums *in vitro*. The inhibition is more effective on the A_2 than on the A_1 isoagglutinins in human serums of Groups B and O. Furthermore, when animal parasites are incubated in Group B human serum the titer of the A_2 isoagglutinins are reduced to negative while the A_1 are not affected. The substance secreted by the animal parasite while incubated in the serum as well as the polysaccharide extracted from the dry worm material are, therefore, related to the A_2 isoagglutinins in the serum (1).

The intravenous inoculation of the ascaris and trichina polysaccharide into monkeys and rabbits also caused an inhibition of the A_2 isoagglutinins in the living animal. This inhibition was complete for a period of 24 hours. Positive titers against the A_2 isoagglutinins were detected on the 48- and 96-hour periods, but these were reduced as compared with the titers of the agglutinins before the animal was treated with the polysaccharide.

The A_2 or anti-O isoagglutinins are irregular agglutinins which react intensely with all erythrocytes of Group O human blood and less intensely with erythrocytes of subgroup A_2. They are sometimes responsible for agglutination of the host's own erythrocytes (autoagglutination) as observed either at 37°C or 6°C. Autoagglutination of erythrocytes, as has been observed during infection with either bacteria or animal parasites, may be partially prevented by the inoculation of the polysaccharide into the animal. The use of the polysaccharide for this purpose would have a logical explanation on the basis first; that the autoagglutinin may have developed as a result of immunization of the host with the isoagglutinogen-like substance in the infective organism and that, therefore, antibodies against the host erythrocytes cause autoagglutination; and second, that the poly-

saccharide when injected as such into animals inhibits the A_2 isoagglutinins.

Circulating autoantibodies have been reported to have a particular significance in acquired hemolytic anemias. Besides present as a circulating antibody, the autoagglutinin also attaches itself to red blood cells. The basic cause for the development of these autoantibodies is still unknown although a large majority give a history of previous infection. The serum of patients with acquired hemolytic anemia usually contains an agglutinin reacting with normal Group O human erythrocytes (2). Whether this agglutinin is the same which agglutinates the host's own erythrocytes, by reacting with some agglutinogen common to the Group O cells and the host erythrocytes, is a point of major significance. Since the anti-O behave similarly to the A_2 isoagglutinins, the role that the isoagglutinogen-like substance of infective agents may have in producing such agglutinins in patients with acquired hemolytic anemia, should be considered. The possibility of inhibiting the agglutinins by injecting the polysaccharides into the patients should be also considered.

The polysaccharides from ascaris and trichina also inhibited to a great extent the agglutinins acting at 6°C. That there was an effect on the agglutinins acting at 6°C irrelevant to those acting at 37°C, is shown by the results in the rabbits whose serum had no agglutinins at 37°C. In these the cold agglutinin titer was reduced following inoculation with the polysaccharides. The role played by cold agglutinins in certain pathological conditions is still not understood. The results of the study also suggest that there is an immunological relationship between the a_2 and cold agglutinins, and therefore, the agglutinogen responsible for both antibodies may be also related.

The trichina larvae and adult pig ascaris from which the polysaccharides can be prepared, are made easily available to the laboratory. A strain of *Trichinella spiralis* can be permanently kept in rats, from which desirable amounts of dried larval worm material can be obtained. Adult ascaris from a pig are easily available from slaughter houses.

Summary. 1. Monkeys and rabbits were injected intravenously with various amounts of polysaccharides (dissolved in 0.85% saline) prepared from the muscle stage of *Trichinella spiralis* and from adult *Ascaris lumbricoides* from pig. 2. The titer of the a_2 isoagglutinins detected at 37°C in the serums of the animals was reduced to negative for a period of 24 hours, and remained lower than the titer before inoculation for a period of 96 hours. 3. The titer of the a_1 was also reduced, but not as much as the a_2 agglutinins. 4. The titer of the agglutinins acting at 6°C against A_1, A_2 human, sheep, and the animal's own erythrocytes was also reduced after inoculation of the polysaccharide. 5. The possibility of injecting these polysaccharides to inhibit autoagglutination of erythrocytes as seen in certain pathological conditions is mentioned.

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Enhancement of Canine Isohemagglutinin A by a Heat Labile Serum Factor, Probably Complement.* (20180)

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The demonstration by Young and his colleagues(1) of a variety of isohemagglutinins in the dog, which in many respects mimic those observed in humans, has facilitated *in vivo* investigation of immune hemolysis, heretofore limited by the inability to study adequately such reactions in human subjects. Sporadic reports of canine isoantibodies have appeared in the literature in the past and are adequately reviewed by Hamilton(2) and Young(1). Young and his co-workers(3) have reported 6 canine agglutinogens A, B, C, D, E, and F. These do not correspond to human agglutinogens of similar designation. The incidence of these agglutinogens and their corresponding agglutinins are summarized by Christian(4) and Young(3). Hemolytic reactions following the transfusions of incompatible blood or plasma and hemolytic disease of the newborn have been produced readily with anti-A but have not been reported with other canine isoantibodies(5).

The A agglutinogen or its variant A′ has been found in 63% of a large group of random dogs and has been demonstrated to be strongly antigenic, in most instances producing high titre anti A within 8 to 21 days(3,4). *In vitro* anti-A acts as a specific hemolysin as well as an agglutinin; is thermostable; most active at 37°C; fixes complement; and sensitizes red cells for the antiglobulin reaction(4,14). An apparently unique characteristic of canine anti-A is the enhancement of its agglutinative capacity by a heat labile factor in normal dog serum. Moreover, fixation of complement in normal fresh canine sera by an antigen-antibody precipitate destroys the capacity of such sera to enhance the antibody. The addition of guinea pig complement, however, does not restore this activity(4). Likewise human and bovine albumin have been ineffective(4). Initial studies on the nature of this heat labile factor in normal dog serum are the subject of this report.

Methods and material. Of the anti-A sera used throughout this study, 5, (6, 16, 22, 65 and 75) were produced in this laboratory by multiple small transfusions (5 cc) of A cells into A negative dogs. A sixth sera (49A) was kindly supplied by Drs. L. E. Young and W. A. O'Brien of the University of Rochester. Anti-dog serum rabbit serum for use in the antiglobulin test (Coombs) was prepared as described by Christian *et al.*(4). Pooled human, guinea pig sera (from this laboratory) and lyophilized commercial guinea pig serum (lyocomplement, Sharp and Dohme) were absorbed with pooled canine cells prior to use. Fresh bovine sera‡ were absorbed 3 times at 4°C with pooled A cells prior to use. Sera from 12 steer as well as a pool of bovine sera (from 6 steer) were prepared. Thirty per cent commercial albumin and 25% human albumin (National Red Cross) were used as indicated. Complement and its component parts were inactivated as follows: C′1, 2, by heating at 56°C for 30′; C′3 by an insoluble, carbohydrate fraction of yeast, zymosan, as described by Pillemer(6); C′4 was inactivated by incubation at 25°C with .15 N NH_3, as cited by Kabat and Mayer(7). Mid-piece (C′1 and 3) and end-piece (C′2 and 4) were separated by dilution with 10 volumes of M/200 KH_2PO_4 at 4°C. The precipitate thus formed was redissolved in 5 volumes of 0.9% NaCl and the pH adjusted to 7.3 with .1 N $NaHCO_3$ the supernatant was made isotonic with 10% NaCl and pH adjusted to 7.3 with N $NaHCO_3$. The final dilution of mid-piece was 1:5 and end-piece was 1:12. Alternatively,

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separation was produced by dialysis against 4 liters of distilled H_2O at 4°C, or phosphate buffer of pH 5.4 and ionic strength .02(7). Dialysis was continued over an 8-hour period with 2 changes of the bath. An increase in dialyzing surface was obtained by the insertion of a pyrex tube within the cellophane dialysis bag as suggested by Bier(8). Following the dialysis the precipitate was washed twice with phosphate buffer and restitution of pH and tonicity was effected as in the dilution method. Final dilutions were 1:10 for end-piece and 1:5 for mid-piece. Since results obtained with both methods were identical, no distinction is made in the text. Complement fixation was obtained by the use of an antigen-antibody precipitate using anti-dog-rabbit serum and dog serum. Alternatively, fixation was produced with anti-bovine dog sera from an immunized animal and bovine cells. Complement fixation was carried out at 4°C. Sera were checked for residual C′ with a sheep cell indicator prior to use. All antisera were inactivated at 56° for 30′ prior to use and dilutions were made with saline§ throughout. Preliminary studies indicated that there were no significant differences between titres obtained with heated antisera diluted with saline and those using heated antisera and fresh normal dog serum as a diluent, provided that fresh serum is subsequently added to the system. Christian *et al.* reported a similar experience (4). Immune sera were stored at –20°C when not used the day of collection as was absorbed human, guinea pig and bovine sera. Canine A blood was collected without anticoagulant, the serum separated and the cells washed from the clot. Prior to use all cells were washed three times in 0.9% NaCl and resuspended in sufficient buffered saline to make approximately a 4% suspension. Titrations were performed using a total volume of 0.2 cc consisting of .05 cc of antisera; .05 cc of saline suspended A cells; .05 cc of each of two substances to be tested. When only one substance was tested the additional .05 cc consisted of buffered saline. All titrations were incubated at 37°C for 15′ and centrifuged at 300 G for 30″. Antiglobulin tests (Coombs) were performed as described by Christian(4). The degree of hemolysis is indicated by numerals 0-4; where 4 is complete hemolysis, 3 marked hemolysis, 2 moderate hemolysis and 1 slight hemolysis. Agglutination is indicated by +'s with ++++, a solid clump of cells; +++, large clumps of cells; ++ medium clumps of cells; + small clumps of cells giving a granular appearance; ± microscopic agglutination without visible macroscopic agglutination.

Results. Preliminary studies of the characteristics of anti-A, including observations on thermal range, hemolytic activity, complement fixation, sensitization for antiglobulin reaction, and serum enhancement, were in agreement with those reported by Christian(4). It was noted that high titre anti-A sera were capable of producing agglutination in the first dilutions in the absence of the serum factor. Maximal agglutination and hemolysis were lacking in such systems. Weak anti-A sera were incapable of agglutinating A cells in the absence of the enhancing factor. It also was demonstrated that there was a wide individual variation amongst A cells both in agglutinative capacity and susceptibility to hemolysis. Consequently, Christian(4) has subdivided the A agglutinogens into two groups, A and a weak variant A′. We have observed, as did Christian, a broad spectrum of activity within each group. These variants are due to differences in the agglutinogens rather than in content of serum enhancing factor as is shown by similar hemolytic and agglutinating titres obtained with homologous as well as autogenous sera, an example of which appears in Table I.

The most unique characteristic of canine anti-A is the enhancement by fresh canine sera. Table I demonstrates that this effect differs from the usual incomplete or univalent isohemagglutinin. The latter generally manifest a similar degree of activity in fresh or heated serum, albumin and albumin-serum mixtures. There is, however, a marked reduction in the activity of canine anti-A when heated serum or saline replaces fresh serum in the reaction. The addition of human or bovine

§ Following demonstration of the importance of divalent cation in the reaction, studies were repeated using a veronal-bicarbonate buffered saline with optimal Mg^{++} and Ca^{++} added(9). Unless otherwise indicated, saline in this paper refers to buffered saline with added Mg^{++} and Ca^{++}.

TABLE I. Comparison of Titres of Anti A Serum against A Cells in Presence of Fresh Serum with Those Obtained in Saline, Heated Serum, Albumin and Serum-Albumin Mixtures.

Reagent 1	Reagent 2	Final dilution of anti A serum (49A) 4	8	16	32	64	128	256	512
U. A. S.*	Saline	4	4	3+	2+++	++++	++	±	—
U. H. S.	"	4	4	3+	2+++	+++	++	±	—
U. A. S.	"	++	+	±	—	—	—	—	—
B. Al.	"	++	++	+	—	—	—	—	—
Hu. Al.	"	++	++	±	—	—	—	—	—
U. H. S.	B. Al.	4	3++	1+++	+++	+++	+	—	—
H. A. S.	"	+++	++	+	±	—	—	—	—
Saline	Saline	++	+	—	—	—	—	—	—

* U. = unheated; A. = autogenous; S. = serum; H. = heated; B. Al. = bovine albumin; Hu. Al. = human albumin.

Degree of hemolysis is expressed numerically (4–); agglutination by +'s.

albumin-serum mixtures to such reactions has failed to produce significant enhancement in our experience.

The observations, that inactivation of fresh sera by heating at 56° for 30′ or fixation of complement destroys the enhancing effect of fresh serum, suggest that complement is involved. Accordingly, the effect of inactivation of specific fractions of complement, separation of mid-piece and end-piece, and complement fixation were studied. A typical protocol is shown in Table II. It may be noted that such procedures resulted in complete loss of enhancing effect. It is difficult to establish with certainty from these preliminary data whether C′ or one or more of its fractions is required for this reaction, since the method of inactivation while specifically destroying one fraction may also significantly reduce other components(7). It seems likely however, that the entire C′ rather than one or more of its components is necessary for the manifestation of maximum canine anti-A activity.

TABLE II. Effect of Inactivation of Complement (C′), Its Components and the Binding of MG^{++} on the Reaction of Anti A and A Cells.

Reagent 1	Reagent 2	Final anti A (#22) titre†
Unheated S.*	Saline	256
Heated S.	"	16
NH_3 inactivated S.	"	8
Yeast inactivated S.	"	8
Mid-piece	"	4
End-piece	"	8
C′ fixed S.	"	8
Unheated S.	.15M citrate	16

* S = serum.

† Inactivated sera—1-2 + agglutination at 1:2; unheated serum, complete hemolysis at 1:8; partial hemolysis at 1:32; ++++ agglutination at 1:64.

Further evidence of the role of complement in this reaction is provided by the effect of Mg^{++}, a divalent cation essential for optimum complement activity(10). Binding Mg^{++} by a .15 Molar concentration of citrate effectively reduce the activity of anti-A sera (Table II). Restoration of free Mg^{++} by the addition of excess Mg^{++} as $MgCl_2$ restores this activity. It has also been shown that removal of Mg^{++} by dialysis, binding with phosphate or the sodium salt of ethylene diamine tetracetic acid, a chelating agent, at pH 7.1 will influence the anti-A reaction in a similar fashion. Moreover, if an excess of Mg^{++} is introduced into fresh serum there will be a reduction in activity, although not of the magnitude noted with binding of Mg^{++}. These observations are in keeping with those of Mayer *et al.* on the effect of the magnesium ion in the complement reaction(10).

A variety of animal sera have been studied to observe whether fresh heterospecific sera are capable of producing maximum anti-A activity. Extensive studies have been carried out on absorbed guinea pig complement and human sera. These sera, while capable of producing hemolysis in the lower dilutions of anti-A, do not demonstrate the agglutinative activity noted with fresh canine sera. Limited observations on rabbit, horse and swine sera have thus far been negative. In bovine sera, which lack lytic activity with most antigen-antibody systems(11,12) but have been shown to have varying degrees of conglutinating complement as well as conglutinin(13), the agglutinative capacity of anti-A is equivalent to that observed in fresh canine sera.

TABLE III. Effect of Bovine Serum on the Enhancement of Canine Anti A.

Serum added*	Final anti (#6) titre
Unheated canine	256§
Unheated B.†	512‖
Saline	4¶
B. serum‡	256‖
Heated B.	8¶
NH_3 inactivated B.	8¶
Yeast inactivated B.	8¶

* Each tube contained .05 cc anti A dilution; .05 cc saline, and .05 cc serum.

† B = bovine serum.

‡ Unheated bovine serum added to washed cells from saline titration in above row.

§ Complete hemolysis at 1:8; partial hemolysis at 1:64; 4 + agglutination at 1:64.

‖ 4 + agglutination — at 1:128, hemolysis absent in all dilutions.

¶ Maximum agglutination was 1 — 2 + at 1:2.

TABLE IV. Effect of Fresh Canine, Bovine and Antiglobulin Sera on the Reaction of Anti A and A′ Cells.

Serum added	Final titre anti A (#6)
Unheated canine	4*
Antiglobulin	128†
Unheated bovine	4*
Antiglobulin	128†

Antiglobulin serum was added to washed cells from row immediately above it.

* 1+ agglutination — no hemolysis.

† 4+ agglutination at 1:8; 3+ at 1:32.

Pooled absorbed sera and ten individual samples have yielded similar results when tested against six different anti-A sera. A typical protocol is presented in Table III. One further sample of fresh bovine serum was ineffective when added to the reaction alone, but when end-piece or heat inactivated dog sera was added, typical enhancement was noted. Another serum failed to demonstrate the enhancing effect even in the presence of heated canine serum. The agglutination of A′ canine cells in bovine sera is similar to that observed with fresh dog sera and weaker than with antiglobulin serum (Table IV). Inactivation of bovine sera by heat, zymosan or NH_3 or binding of Mg^{++} results in loss of enhancement activity (Table III). It could be demonstrated, with these two exceptions, that bovine sera contained all the factors necessary for enhancement by incubating inactivated sera and A cells in a saline titration, washing 3 times and resuspending in bovine sera with results as depicted in Table III. This indicated that the heat labile enhancing substance is not involved in the union of antigen-antibody but rather in a physical manifestation of this union. This concept is supported by the effect of antiglobulin(14). If anti-A titrations are made in saline or inactivated sera, weak agglutination results. When the cells from the above are washed and absorbed anti-dog serum rabbit serum (Coombs) is introduced, agglutination equivalent to or greater than that observed with fresh serum occurs (Table V) indicating that the cells were sensitized in the absence of complement.

Discussion. Enhancement of activity by a heat labile component of serum is apparently unique to canine anti-A amongst erythrocyte isoantibodies. Data have been presented in an effort to clarify this phenomenon. It has been demonstrated that fixation of complement or inactivation of its component parts results in a decrease in anti-A activity. Moreover, the concentration of magnesium, a cation essential for complement activity, influences this reaction. Guinea pig, human, and in limited studies, rabbit, equine and swine sera, did not manifest the enhancing activity noted in canine sera, although, with the exception of equine sera, hemolysis was increased in an otherwise inactivated system. Bovine serum, although hemolysis is not present, does contain a heat labile component capable of restoring maximal agglutination. This would suggest that the canine anti-A reaction requires complement and may be analogous to the conglutinin phenomenon recently reviewed by

TABLE V. Demonstration by Antiglobulin Technic of Sensitization of A Cells by Anti A Sera in Presence of Inactivated Complement.

Treatment of reagent sera	Titre of anti A sera (dog #6 GT) Initial	Antiglobulin
Fresh sera	256	1024
Saline	16	512
Heated	16	512
NH_3 treated	8	512
Yeast treated	8	512
Mid-piece	16	512
End-piece	16	512

Cells from initial titration washed 3 times in saline and subsequently incubated with antiglobulin serum.

Hole and Coombs(15). This reaction differs from the albumin-serum effect on incomplete Rh antibodies erroneously designated conglutination by Wiener(16), which does not require fresh serum or complement. It also differs from the Donath-Landsteiner reaction where C'4 is necessary for union of antibody and antigen(17). In the canine anti-A system sensitization of cells occurs in the presence of inactivated sera as may be demonstrated by the subsequent addition of antiglobulin (Coombs) sera or fresh sera to the sensitized cells.

The possibility that this serum effect is conglutination raises many questions. Streng (18) was unable to demonstrate conglutinin in canine sera using a conventional sheep cell system. Many investigators have failed to detect conglutinating as well as hemolytic complement in bovine sera. Most of these observations were carried out using a sheep cell-anti-sheep cell indicator. Rice(19) however, has recently demonstrated that complements and conglutinins are not mutually interchangeable and will vary in reactivity with the type of indicator used. Moreover, she has detected both hemolytic and conglutinative complement in bovine sera if anti-rabbit cell sheep serum is used as antibody and rabbit cells as antigen in the indicator system(19). Although deficiencies might exist in individual animals, it would certainly seem unreasonable to assume that one or more species lack such a ubiquitous protein complex as complement. It seems more likely that with the use of other antigen-antibody complexes, complement and conglutinin may be demonstrated in species, which heretofore, have been considered devoid of these substances. Accordingly, quantitative studies are being conducted on reactivation of dog complement and the interchangeability of complement fractions and conglutinin of various species utilizing the canine anti-A system.

Summary. Data have been presented suggesting that the enhancing effect of a heat labile component of canine serum on the agglutinative capacity of the canine isohemagglutinin A is due to complement. Although guinea pig and human serum are ineffective, bovine serum, in most instances, will restore maximum activity to a previously inactivated system. Further data suggest that this enhancement, which appears to be unique so far as erythrocyte isoantibodies are concerned, is related to conglutination.

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A Microbiological Approach to Nutritional Evaluation of Proteins. (20181)

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The accepted methods for the determination of the nutritional value of proteins are based on a variety of physiological responses of common laboratory animals (mice, rats, dogs, etc.). All these assays are time-consuming, laborious and quite expensive, hence, that attempts are being made to devise more convenient methods. Melnick and Oser(1) showed that the rate of digestion of proteins with pancreatin, as measured by formal titration, is influenced by heat processing of the protein. This correlation was the basis of an *in vitro* method for estimating susceptibility of proteins to enzymic digestion. Anderson and Williams(2) used the growth response of the proteolytic ciliate, *Tetrahymena geleii,* towards different proteins as a measure of their biological value. These workers succeeded in overcoming certain technical difficulties encountered by earlier investigators (3,4) by incorporating 2,3,5-triphenyltetrazolium chloride into the medium. This compound is reduced by the protozoan to the red triphenylformazan, and is suitable for colorimetric determination despite the turbidity of the protein suspension.

It occurred to us that digestion of proteins with pancreatic enzymes, would yield hydrolysates which could be tested for their growth-promoting activity on a suitable test organism in a medium devoid of the 10 essential amino acids. The growth response would thus be determined in a single assay by the particular amino acid which appears in the mixture in a limiting concentration. Pancreatin, consisting of a mixture of most of the enzymes involved in the decomposition of proteins by higher animals and man, seemed a suitable digestive agent.

Experimental. Procedure. The method is carried out in 4 stages: a) The protein suspension is hydrolyzed with pancreatin. b) The growth-promoting activity (biological value?) of the hydrolysate on *Streptococcus faecalis,* in a medium devoid of the 10 essential amino acids, is tested; the growth response, in a single run, is determined by that essential amino acid which is present in limiting concentration. c&d) The actual composition of the hydrolysate with respect to the essential amino acids is determined and the effect of supplementing the limiting amino acids is analyzed. *Hydrolysis.* The enzymatic hydrolysis is carried out according to Melnick and Oser(1). To allow better contact between enzyme and substrate the digestion was carried out under constant shaking by means of a magnetic stirrer. Five g of a protein sample is suspended in 150 ml of alkaline buffer (pH 8.4), containing 300 mg of 3 U.S.P. Pancreatin and covered with a layer of toluene. The digestion is carried out for 48 hours at 37°C under constant shaking. About 40% of the protein was hydrolyzed by this treatment. Then, the remaining protein is precipitated by addition of glacial acetic acid and boiling for a few minutes. The supernate is filtered, the pH adjusted to 7.0, and the amount of the amino-N liberated is determined by the formal titration procedure. The solution is finally diluted to contain 1.4 mg of amino N per ml. *Composition of the medium.* The basal medium was prepared according to Henderson and Snell(5), the only important difference being the omission of the essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine). The pancreatic hydrolysates of the different proteins under test serve as the source for the essential amino acids. The original hydrolysate solution (1.4 mg amino N/ml was diluted till a growth response of about 40 to 50% of that of a control was obtained. The controls represent the growth of the test organism in the complete medium of Henderson and Snell(5) without any protein

* Based on data submitted in partial fulfillment of the requirements for the degree of Ph.D.

TABLE I. Comparative Growth of *Streptococcus faecalis* in Pancreatin Hydrolysed Proteins.

	Amino N, γ/ml	Klett reading filter No. 660	Relative activity casein = 100%	Rat growth test (6) casein = 100%
Egg albumin	17.5	100*	105	119
Casein	17.5	95	100	100
Gelatin	60.0	90	29	—
Gluten	87.5	70	15	16
Zein	175.0	20	2	—

* Tests were carried out in the Henderson Snell medium (5), from which the 10 essential amino acids were omitted and one of the protein hydrolysates incorporated. Growth of test organism in the complete medium (5) gives a turbidity reading of 220-250.

hydrolysate added.

Test organism. The organism studied most extensively was a strain of *Streptococcus faecalis*†) which requires all 10 essential amino acids. By arranging the experiment as described, a quantitative evaluation of the nutritive value of the hydrolysate for the given organism is thus obtained. If the protein tested is low in even one of the essential amino acids, or if enzyme treatment fails to liberate enough of an essential amino acid—its growth promoting activity will be low in comparison to others. *Estimation of growth.* The bacteria were grown in regular test tubes containing 2 ml of medium. The bacterial growth turbidity was estimated photometrically using the Klett-Summerson electrophotometer, after incubating the tubes at 37°C for 20-24 hours. The usual growth of the *S. faecalis* in the complete medium gave a reading of about 250, using the No. 660 filter. In cases where rather concentrated solutions of protein hydrolysates, *e.g.*, zein, had to be used, interference in the turbidity reading due to color of the hydrolysate was corrected by adjusting the zero point against an uninoculated tube of a similar sample. Other methods for estimating the bacterial growth may be used as well.

Results. Table I shows the comparative growth response of *S. faecalis* towards pancreatic hydrolysates of 5 proteins. Coagulated egg albumin and gelatin (Difco Laboratories, Inc.), casein and gluten (Nutritional Biochemicals Corporation), and zein (Bios Laboratories, Inc.) were used. As mentioned, this type of experiment was carried out by addition of different quantities of pancreatin treated protein to a medium devoid of the 10 essential amino acids, and the growth compared to that obtained in a complete medium of Henderson and Snell. The protein hydrolysate is usually diluted to give only a 40-50% growth as compared to a control on a complete medium. The readings may thus be made on the steep part of the growth curve and permit a more accurate evaluation of the results. In cases where still lower growth was obtained (a response of 10-30%), the results were interpolated from the curve. The test organism grows almost equally well on both casein and egg albumin (Table I). Gelatin, however, was much less active, the response being about 30% of that of casein. Gluten was even inferior to gelatin; the activity as compared to casein was about 15%. Zein was still less active and at high concentrations the growth was very small; the calculated activity was only about 2% as compared to casein.

A comparison of the results obtained with those given by the rat growth method (6) is shown in the last column of Table I. It will be noted that the agreement between the two methods is very close.

The next question to be studied was the nutritional basis for the results obtained namely which was the amino acid(s) limiting growth in the various protein hydrolysates. In order to obtain this information the deficient medium was supplemented with the usual amounts (5) of only 9 out of the 10 essential amino acids. A different amino acid was omitted in each run. The amount of protein hydrolysate remained as stated above. For the determination of the content of lysine, however, the amounts of both arginine and threonine were reduced from 200 γ per tube

† This strain was obtained through the courtesy of Dr. B. Volcani, The Weizmann Institute of Science, Rehovoth, Israel.

TABLE II. Effect of Supplementation with Essential Amino Acids on the Growth Response to Pancreatin Treated Proteins.*

Deficient medium	Egg albumin, 17.5 γ/ml amino N A	Casein, 17.5 γ/ml amino N B	Gelatin, 60 γ/ml amino N C	Gluten, 87.5 γ/ml amino N D	Zein, 175 γ/ml amino N E	Gluten‖ heated, 115 γ/ml amino N F	Gluten‖ heated with glucose, 175 γ/ml amino N G
a. 10 essential amino acids missing	100†	95†	95‡	70†	20†	100§	30§
b. Plus 9 essential amino acids Amino acid omitted:							
Arginine	150	145	195	270	250	190	170
Histidine	150	240	200	260	240	210	165
Isoleucine	180	185	170	260	240	210	170
Leucine	210	250	155	260	240	210	165
Lysine	125	135	190	65	15	95	35
Methionine	250	240	165	260	230	220	175
Phenyl alanine	240	240	195	240	230	190	170
Threonine	165	180	165	260	230	185	165
Tryptophane	250	240	105	260	250	210	175
Valine	210	210	200	260	240	185	165
c. Supplemented with:							
Arginine, histidine, isoleucine, lysine, and threonine	240						
Arginine, isoleucine, lysine, and threonine		250					
Lysine				270	260		
Tryptophane			165				
Isoleucine, leucine, methionine, threonine, and tryptophane			210				

* Test organism used: *S. faecalis*. Numbers represent Klett turbidity readings. Growth in complete Henderson-Snell medium (without protein hydrolysate added) was: † 250; ‡ 225; § 210.
‖ Samples treated for 2 hr at 15 lb pressure before enzymatic digestion.

to 20 γ per tube. These changes were introduced in order to avoid inhibition of the utilization of the lysine of the hydrolysate.‡

It is evident that the limiting factor in the egg albumin preparation is lysine (Table II-A). When all amino acids except lysine were added to the protein hydrolysate, growth increased only slightly (from 100 to 125) on the turbidity scale. Addition of lysine alone did not restore full growth. Here arginine and histidine became limiting (the reading was 150 in the absence of either arginine or histidine). Other amino acids appearing in limited concentration were threonine, isoleucine, leucine and valine. Tryptophane and methionine were found in excess; the omission of either does not affect the full response. Supplementation of the egg albumin sample with the missing amino acids (Table II-A, c) brings up the growth to that of the control (Table II, footnote 2). Column B shows similar results for casein. In this case the number of limiting amino acids is restricted to lysine, arginine, isoleucine and threonine, the first two being the more important ones. Using gelatin, the main limiting amino acid was tryptophane (Column C). Supplementation with tryptophane alone (C, c) was insufficient to secure full growth, as leucine, isoleucine, methionine and threonine were also limiting. The low activity of gluten was due to the low content of lysine; supplementation with lysine alone resulted in a full response (Column D). Essentially the same is true for zein (Column E).

Effect of heating with glucose. Heating the protein samples in presence of glucose before

‡ Grossowicz, N., and Halevy, S., unpublished data.

enzymic hydrolysis reduces markedly the growth of *S. faecalis*. A sample containing 6 g of gluten and 5 g of glucose was moistened with 2.5 ml of water and autoclaved for 2 hours at 15 lb pressure. A control sample without glucose was similarly treated. The growth responses to the untreated versus the glucose heated samples of gluten, are shown in columns F and G, respectively (Table II). It will be noted that heating with glucose prior to enzymatic treatment reduced the response to 20%. This deleterious change was due to the reduction in available lysine; the concentration of all the other amino acids became limiting, too, but to a lesser extent.

Discussion. Chemical hydrolysis of proteins with strong acid or alkali provides a mixture of amino acids the composition of which has been estimated by numerous investigators quite accurately using microbiological, chemical, or physical methods. The methods provide, at the best, data from which the theoretical composition of amino acids of various proteins could be estimated. None of them, however, give any clear idea of the nutritional value of the proteins in question. For this purpose various kinds of biological assays are being used. The reason for using these methods (growth or gain of weight of young animals, maintenance in adult animals, nitrogen balance tests, etc.) is that the biological value of a given protein depends not only on its composition in amino acids, but, among other factors, also on the extent of the liberation of the different amino acids during the process of digestion (enzymatic hydrolysis). In the body not only the composition, but also the physical state of the protein, for example whether heated or not, plays an important role. The idea was therefore advanced that treatment of proteins with a mixture of the enzymes normally active for this purpose in the body in the form of pancreatin, would provide an hydrolysate containing the essential amino acids in their "biological ratio," capable of being assayed microbiologically. A correlation, however, with one of the common biological tests would depend on whether or not a suitable organism was found.

The results presented (Table I), although based on a few samples only, show such a correlation for 3 of the 5 proteins tested. There is good agreement for casein, egg-albumin and gluten between results obtained by the rat growth method(6) and the pancreatin treated samples as assayed with *S. faecalis*. As for the 2 remaining proteins, the low value for zein seems correct, although comparative data are missing. The results for gelatin, however, appear too high in comparison with the rat growth test(7).

Our tests showed that the low biological value of both gluten and zein were due to low lysine, while gelatin was a poor protein on account of being limiting in tryptophane—findings which are in accordance with those obtained by other methods(7). A few discrepancies were also found while the nutritional value of egg-albumin and casein rated as high as in the rat growth test(6), both proteins were found to be deficient in lysine by our test. On the other hand, according to other methods, the limiting factor in egg-albumin is isoleucine and in casein it is methionine(8). A possible explanation for the differences obtained with our method may be due to an exaggerated requirement of lysine by *S. faecalis* in comparison to the rat. Another possibility, however, should not be overlooked. The rate of utilization of a given amino acid may depend on its quantitative relationship or balance towards the other amino acids present. Excess of arginine or threonine or both may inhibit markedly the utilization of lysine present in casein and egg-albumin hydrolysates.‡ The method appears, therefore, very suitable for studying imbalances among amino acids and their effects upon the nutritional value of the different proteins.

The nutritive value of a given protein may vary according to the criteria employed; using the maintenance method, wheat gluten appears to be as good a protein as beef muscle, while for growth the latter is definitely superior(9).

Whereas our results seem to agree with those obtained by the rat growth method(6), it appears possible that other micro organisms may follow the patterns obtained with some of the other biological methods (maintenance,

repletion, etc.). This possibility is now being investigated.

Summary. 1. A microbiological method to assess the biological value of proteins is described. It implies the digestion of proteins with pancreatin and subsequent testing of the growth-promoting activity of the hydrolysate for *S. faecalis,* in a medium devoid of the 10 essential amino acids. The nutritive value of the protein digest is determined in a single assay by the amino acid liberated in a limiting concentration. 2. The results obtained with casein, egg albumin, gelatin, gluten, and zein are presented and their agreement with the biological values obtained by the rat growth method is suggested. 3. The growth promoting activity (biological valued) of casein and egg albumin is high and about equal; gelatin and gluten, in comparison, are much less active, 29 and 15%, respectively, and zein still less (2%). 4. At the limited growth level, casein is deficient in: lysine, arginine, isoleucine, and threonine. Egg albumin under comparable conditions is limited in lysine, arginine, histidine, threonine, isoleucine, leucine, and valine. Gelatin is low in tryptophane, isoleucine, leucine, methionine, and threonine. Gluten and zein are short in lysine. 5. The suitability of the method described for studying imbalances of amino acids is proposed.

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Induction of Diabetes Insipidus in Adrenalectomized Dogs with Cortisone.* (20182)

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Large daily doses of DCA, when administered to intact dogs over a sufficiently prolonged interval, induce a syndrome of polyuria and polydipsia apparently identical with that of diabetes insipidus. According to Ragan *et al.*(1), pituitrin is relatively ineffective in controlling the polyuria and fluid restriction does not cause dehydration. Mulinos *et al.*(2) observed that withdrawal of salts from the diet reduced the severity of the polyuria and that large doses of pitressin in oil led to reduction of water intake and urine volume.

Recently it has been shown that daily injections of cortisone given to normal dogs for periods ranging from 2-3 weeks also bring about a marked polyuria and polydipsia(3,4). Sirek and Best(4) report that 10 I.U. of posterior pituitary hormone administered to one dog for one day restored almost normal conditions so far as thirst, polyuria and urine specific gravity were concerned.

Material and methods. Four mongrel male dogs were used in the present experiments; they had been adrenalectomized for periods varying from 8 months to 2 years. The animals were kept in metabolism cages and fed a special diet consisting of Warner's dog food,

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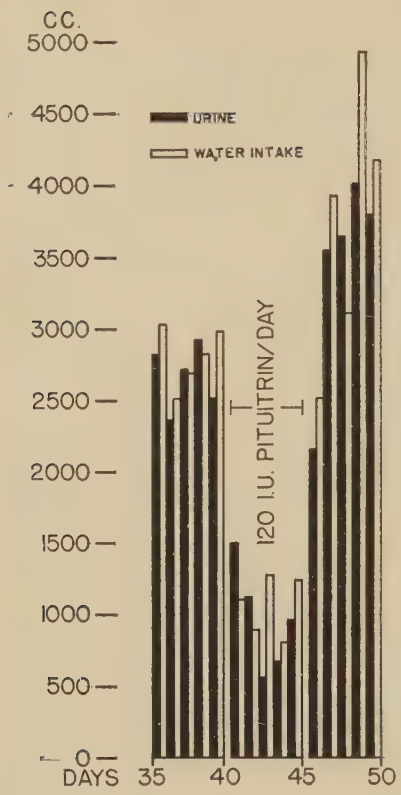

FIG. 1. Control of polyuria and polydipsia with pituitrin. Experiment started on 36th day of cortisone treatment.

horse meat and tripe. All necessary vitamins were added plus one teaspoonful of yeast. The daily ration (435 g, wet weight) contained 0.93 g of Na and 0.94 g of K. Water to the extent of 145 cc was added for mixing the food into a coarse mush. The water drunk and the urine voided during each 24-hour interval were measured and due allowance made for any evaporation and also for the water included in the food. The various methods employed for determining urine electrolytes are: Na(5), Cl(6), K(7). A fore-period of 5 or 6 days was allowed each dog when first placed in the metabolism cage; during this time the animals were fed the low Na and K ration and received the usual maintenance dose of 0.5 mg/dog/day of DCA. The control values for the various urine constituents were determined during this interval. The figures in Table I for water intake and output and urine electrolytes each represent weekly averages of the 7 daily 24-hour specimens. A microcrystalline suspension of cortisone-acetate (Merck's cortone) was administered once daily, intramuscularly, in doses of 10 mg/kg/day.

Results. Polyuria and polydipsia. Marked polyuria and polydipsia occurred within the first 10-14 days of cortisone therapy. Representative data, obtained from 2 of the 4 dogs studied, are given in Table I. Within the first 21 days the amount of urine, voided each 24 hours, was 5-10 times that eliminated during the same time interval of the control period. The maximum quantity of urine for any single 24-hour period passed by each of the 2 dogs listed, was as follows: Dog 1, 1790 cc; Dog 2, 2795 cc. Following withdrawal of cortisone at the end of the 6th-7th week, a sharp decline in water intake and urine output took place (Table I). The animals were not permitted to develop severe symptoms of adrenal insufficiency; when the blood pressure had declined to 80-90 mm Hg and the serum Na to values between 130-135 meq/1, the experiment was terminated. It usually required 20-30 days after discontinuing the cortisone before symptoms appeared. Apparently this length of time is required for complete absorption of the considerable amount of microcrystalline material injected.

Electrolytes of the urine. Daily urinary excretion of Na and Cl increased under the stimulus of the large doses of cortisone during the period of profound polyuria (Table I). However, in this same interval renal elimination of water increased 5-10 fold per 24 hours; hence, the dogs were excreting a highly dilute urine insofar as Na and Cl were concerned. When cortisone was withdrawn, an increase in the daily excretion of both of these electrolytes took place. The renal excretion of K was studied in but 2 animals (No. 1 and 2, Table I). In both there was an increased K elimination during the first 4 weeks after cortisone administration, but this was not sustained throughout the entire period of treatment. During the last 2-3 weeks, previous to termination of the experiment, urinary excretion of K declined to a value approximately that of the control fore-period.

Control of the diabetes insipidus by posterior pituitary extract. An attempt was made

TABLE I. Electrolyte Excretion of 2 Adrenalectomized Dogs Exhibiting a Diabetes Insipidus-Like Syndrome Due to Injecting 10 mg Cortisone per kg/day.

Wt, kg	Exp. period, wk	Urine electrolytes, avg meq/24 hr sample			Avg urine vol, cc/day	Avg water intake, cc/day
		Na	Cl	K		
10.7	1*	31.8	26.2	13.5	146	226
	1†	37.6	41.2	29.0	341	364
	2	46.1	47.5	19.7	934	965
	3	49.4	52.6	17.6	866	997
	4	46.7	56.4	17.1	1386	1565
	5	48.3	52.3	13.4	1199	1350
	6	40.3	45.8	15.7	923	1045
	7	41.6	49.3	13.7	1139	1257
	1‡	56.2	65.3	×	683	705
	2	48.7	55.2	×	516	591
	3	52.7	54.5	×	708	797
12.7	1*	30.9	31.7	17.5	195	403
	1†	49.4	62.8	29.9	1049	1025
	2	55.5	64.5	19.6	2036	2141
	3	43.5	46.3	19.2	1206	1305
	4	66.8	67.7	20.0	1301	1367
	5	63.8	68.6	15.6	1215	1275
	6	78.6	79.1	14.5	1202	1226
	1‡	80.4	85.0	×	956	1140
	2	92.8	90.9	×	585	693
	3	81.1	78.7	×	507	675

* DCA fore-period. † Cortisone started.
‡ Cortisone discontinued. × = not determined.

to control the polyuria and polydipsia of 2 animals by pituitrin. The pertinent data obtained from study of one dog are shown in Fig. 1 and they reveal the striking effect of posterior pituitary extract on the syndrome. The average daily urine volume for 5 days preceding pituitrin injection was 2672 cc; whereas, it promptly declined to an average of 971 cc during the 5-day subcutaneous administration of 120 I.U. of the pituitrin in divided doses of 40 I.U. each. After withdrawal of the extract the average daily urine volume rose to 3432 cc. Smaller daily doses of 5-20 I.U. of pituitrin, twice each day, had little or no effect upon the polyuria and polydipsia of Dog 3 (not listed in Table I). However, 40 I.U., 3 times daily, exerted a noticeable effect.

Discussion. Britton and co-workers(8,9) were the first to advance the theory that a normal water balance is maintained owing to a physiological antagonism between the diuretic action of adrenal cortical hormones and the antidiuretic action of posterior pituitary hormone. Recent work by Gaunt, Birnie, and Eversole(10) and others have tended to substantiate the main points of the theory. Thus, when large doses of cortisone (or DCA(1,2)) are administered, they greatly enhance urine volume by 1) augmenting thirst due to Na retention; 2) inhibiting tubular reabsorption of water; and 3) probably by reducing greatly the amount of antidiuretic hormone released by the posterior pituitary. Hence, the diabetes insipidus is due to an upset in the physiological balance between the 2 antagonists controlling water balance. Since cortisone in massive doses acts as a diuretic, it would presumably overwhelm the antidiuretic function of the posterior pituitary and eventuate in a marked polyuria and polydipsia. Factors other than those mentioned are also probably involved, but since they are unknown, it is futile to speculate concerning them.

Summary. High cortisone dosage in adrenalectomized dogs induces marked polyuria and polydipsia. The 24-hour urine volume may exceed control values by 5-10 fold. The daily renal excretion of Na, Cl, and K is increased, but considering the quantity of water eliminated, the urine is very dilute. Pituitrin is effective in controlling the polyuria and polydipsia only when administered in large amounts. The normal water balance was not re-established until long after cortisone injections were discontinued.

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Evaluation of Agar Dilution Method for Determination of Sensitivity of Bacteria to Antibiotics.* (20183)

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There has developed recently renewed interest in methods of determination of sensitivity of bacteria to antibiotics. This interest has paralleled use of the newer antibiotics and has stimulated the quest for a method for determining quickly the most effective antibiotic in the treatment of an infection. Several methods having the principle of agar diffusion have been used and there has been much discussion concerning each of those methods (1,2). Because of the great practical importance of this problem it was decided to compare an agar dilution and an agar diffusion method of sensitivity determination with the serial broth dilution method, the latter being used as a standard for comparison. An agar dilution method was chosen for evaluation because it offers 4 inherent advantages: 1) it obviates the need for diffusion of the antibiotic to be tested, 2) a direct estimate of the concentration of antibiotic necessary for inhibition of an organism can be obtained, 3) the plates may be prepared in quantity for later use, and 4) a single set of antibiotic dilutions may be used to measure the sensitivity of a number of bacterial cultures.

Materials and methods. Source and identity of bacteria. Ninety-eight strains of bacteria, isolated in the routine diagnostic laboratory serving the University Hospitals of Cleveland, were tested for sensitivity to 6 antibiotics, each organism being tested with all 6 antibiotics by the agar dilution, agar diffusion, and tube dilution methods. The following bacteria were tested: 29 strains of *Escherichia coli,* 20 strains of *Staphylococcus,* 15 strains of *Aerobacter aerogenes,* 9 strains of *Streptococcus,* 9 strains of *Pseudomonas aeruginosa,* 4 strains of *Proteus,* 3 strains of *Pneumococcus,* 3 strains of *Klebsiella pneumoniae,* 2 strains of *Alkaligenes fecalis,* and one strain each of *Salmonella typhosa, Salmonella* sp. (antigenic group C_2), *Shigella paradysenteriae,* and *Shigella sonnei. Culture media.* Tryptose blood agar base medium (Difco) with citrated human blood incorporated in a concentration of 5% was used in the preparation of antibiotic-containing plates. Tryptose agar (Difco) was used in the preparation of plates employed in agar diffusion testing. Tryptose broth was used in the preparation of dilutions used in tube dilution testing. *Antibiotics.* Standard commercial preparations of terramycin,† aureomycin,‡ penicillin, bacitracin, streptomycin, and chloramphenicol were employed. Unbuffered crystalline aureomycin hydrochloride was used for aureomycin determinations. For tests involving the agar diffusion method, commercially prepared antibiotic discs were used ("Dia-Discs," Commercial Solvents Corporation); the disc having the greater quantity of antibiotic was used routinely. *Agar dilution method.* The agar to be used was melted and then cooled to 45°C. An antibiotic was mixed with the agar and plates were poured. The various antibiotics were used in concentrations of 20, 5, 1.25, .312, and .08 units (or mcg) per ml. All plates were stored in a refrigerator at 4°C until they were used. In this test, a sector of the surface of each of a series of plates was streaked with a 10^{-3} dilution of an 18-hour broth culture of the organism to be tested; 6 to 8 different cultures were placed on each plate. After overnight incubation at 37°C the plates were examined. Complete inhibition of growth was chosen as the end-point.

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† Terramycin was kindly supplied by Dr. Alan Wright, Medical Director of the Chas. Pfizer & Co., Brooklyn, N. Y.

‡ Aureomycin was kindly supplied by Dept. of Clinical Research, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Agar diffusion method. In this test the surface of a tryptose agar plate (blood agar if a *Streptococcus* was being tested) was heavily inoculated with the organism to be tested, antibiotic-containing discs were placed on the surface, and the plate was incubated at 37°C. After overnight incubation the plate was examined and, if growth of the organism had been inhibited, the diameter of the zone of inhibition was recorded. *Tube dilution method.* Five concentrations of an antibiotic were prepared by dilution in tryptose broth. To 0.5 ml quantities of the antibiotic-containing broth were added 0.5 ml quantities of a 10^{-3} dilution of an 18-hour broth culture of the organism to be tested. The final concentrations of the antibiotic were 20, 5, 1.25, .312 and .08 units (or mcg) per ml. Citrated human blood was incorporated in broth used in the dilution of cultures of streptococci. The tubes were examined after overnight incubation. Complete inhibition of growth, indicated by absence of visible turbidity, was selected as the end-point.

Experimental results. A total of 98 bacterial strains, of 13 genera, were tested for sensitivity to penicillin, bacitracin, streptomycin, chloramphenicol, aureomycin, and terramycin by 3 methods currently in use, *viz.* agar dilution, agar diffusion, and tube dilution. The results of simultaneous tests of sensitivity to 3 of the 6 antibiotics by the agar dilution and tube dilution methods are shown in Tables I-III. It is seen that agreement between the two methods is good, less than 10% of the tests differing by as much as a single 4-fold dilution; in all the determinations, which amount to a cumulative total of 1176, there are only 5 instances in which there is a discrepancy greater than a 4-fold dilution.

TABLE I. Comparison of Tube Dilution and Agar Dilution Methods of Testing Sensitivity to Penicillin. Bacteria tested: *Staphylococcus*, 20 strains; *Streptococcus*, 9 strains; *Pneumococcus*, 3 strains.

Inhibiting concentration, tube dilution test (units/ml)	Inhibiting concentration, agar dilution test (units/ml)					
	.08	.312	1.25	5	20	N.I.*
N.I.*						9
20					2	1
5				4		
1.25			1			1
.312						
.08	12†	1	1			

* N.I. = Not inhibited.

† No. of strains, having indicated sensitivity in tube dilution test which were inhibited by given concentration of antibiotic in agar dilution test.

TABLE II. Comparison of Tube Dilution and Agar Dilution Methods of Testing Sensitivity to Streptomycin. Bacteria tested: *Escherichia coli*, 29 strains; *Staphylococcus*, 20 strains; *Aerobacter aerogenes*, 15 strains; *Streptococcus*, 9 strains; *Pseudomonas aeruginosa*, 9 strains; *Proteus*, 4 strains; *Pneumococcus*, 3 strains; *Klebsiella pneumoniae*, 3 strains; *Alkaligenes fecalis*, 2 strains; 1 strain each of *Salmonella typhosa*, *Salmonella* sp. (antigenic group C_2), *Shigella paradysenteriae*, and *Shigella sonnei*.

Inhibiting concentration, tube dilution test (μg/ml)	Inhibiting concentration, agar dilution test (μg/ml)					
	.08	.312	1.25	5	20	N.I.*
N.I.*					2	40
20					16	
5			2	28	2	1
1.25			5	1		
.312		1†				
.08						

* N.I. = Not inhibited. † See Table I.

TABLE III. Comparison of Tube Dilution and Agar Dilution Methods of Testing Sensitivity to Aureomycin. Bacteria tested: See Table II.

Inhibiting concentration, tube dilution test (μg/ml)	Inhibiting concentration, agar dilution test (μg/ml)					
	.08	.312	1.25	5	20	N.I.*
N.I.*						23
20					13	
5			2	33	1	
1.25		1	10	1		
.312		12†	1			
.08			1			

* N.I. = Not inhibited. † See Table I.

In Tables IV-VI a comparison of the agar diffusion and tube dilution test results is presented. For the purpose of tabulation, the results of the tests were used to classify each organism as "resistant," "sensitive," or "intermediate." The concentrations of antibiotic chosen to limit each of these groups was based on the plasma levels of the antibiotic that can be obtained by administration of a reasonable dose of the drug(3-8). Since the blood level that can be obtained varies with each antibiotic, the "yardstick" used to define each of the 3 groups also varies with each antibiotic. The zone diameters chosen to limit each of the groups were based on the over-all range of the diameters of the zones of inhibition produced by an antibiotic-containing disc in the

TABLE IV. Comparison of Tube Dilution and Agar Diffusion (Disc) Methods of Testing Sensitivity to Penicillin. Bacteria tested: See Table I.

Classified by tube dilution test	Classified by Agar Diffusion Test: Sensitive (inhibition zone 20-45 mm)	Intermediate (inhibition zone 8-19 mm)	Resistant (inhibition zone <8 mm)
Sensitive: 14 (inhibited by .08 unit/ml)	12*	1	1
Intermediate: 6 (inhibited by .312-20 units/ml)		4	2
Resistant: 12 (inhibited by 20 units/ml)	2	4	6

* No. of strains, having indicated sensitivity in tube dilution test which were classified as "sensitive," "intermediate," or "resistant" in agar diffusion test (see text).

TABLE V. Comparison of Tube Dilution and Agar Diffusion (Disc) Methods of Testing Sensitivity to Streptomycin. Bacteria tested: See Table II.

Classified by tube dilution test	Classified by Agar Diffusion Test: Sensitive (inhibition zone 22-35 mm)	Intermediate (inhibition zone 12-21 mm)	Resistant (inhibition zone <12 mm)
Sensitive: 7 (inhibited by <5 μg/ml)	4*	3	
Intermediate: 49 (inhibited by 5 & 20 μg/ml)	11	37	1
Resistant: 42 (inhibited by >20 μg/ml)		10	32

* See Table IV.

testing of the 98 strains of bacteria used. There was wide variation in the zones of inhibition produced by the different discs; therefore, the zone diameters that define each of the 3 groups also vary with each antibiotic. The diameters of the zones of inhibition and the antibiotic concentrations of the broth dilutions chosen for division of the organisms into the groups are shown in Tables IV-VI. It can be seen from examination of these tables that in the case of penicillin fair agreement exists between the results of the disc and tube dilution tests. The agreement between results of the 2 tests with bacitracin is also fair. There is poor agreement between the results of the 2 tests with regard to the other 4 antibiotics, however. With aureomycin, while there is agreement between the tests in selecting resistant strains, many were classified as resistant by the agar diffusion method that were classified as sensitive by the tube dilution method. The reverse was true in the case of streptomycin, in that many organisms that were classified as resistant by the dilution method were intermediate as tested by the diffusion method; also, many classified as intermediate by the dilution method were sensitive according to the diffusion method. There was similar rather poor correlation of the results obtained in the testing of chloramphenicol and terramycin.

The effect of the size of inoculum used in agar dilution tests was evaluated by repeating sensitivity determinations using different dilutions of the same broth culture; the dilutions used were 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. In such tests with various strains it was found that the size of the inoculum used did not alter the results.

In order to test the stability of the antibiotic-containing plates the following experi-

TABLE VI. Comparison of Tube Dilution and Agar Diffusion (Disc) Methods of Testing Sensitivity to Aureomycin. Bacteria tested: See Table II.

Classified by tube dilution test	Classified by Agar Diffusion Test: Sensitive (inhibition zone 13-26 mm)	Intermediate (inhibition zone 8-12 mm)	Resistant (inhibition zone <8 mm)
Sensitive: 14 (inhibited by <1.25 μg/ml)	7*	6	1
Intermediate: 48 (inhibited by 1.25 & 5 μg/ml)	8	7	33
Resistant: 36 (inhibited by 20 μg/ml)			36

* See Table IV.

ment was performed. Several series of plates were prepared with each antibiotic and the plates were then stored at 4°C. The plates were streaked with the same strains of bacteria at weekly intervals over a period of 4 weeks and the apparent sensitivity recorded. The aureomycin-containing plates showed no loss in potency during the first 3 weeks; after that period the potency decreased by one-half. The other 5 antibiotic-containing plates that were tested maintained their potency over the full 4-week period.

Discussion. There is evident need in the clinical laboratory for a method of determining quickly, the antibiotic sensitivity of bacteria isolated from infectious processes. The simplicity of the disc method of testing recommends it over the cumbersome tube dilution method. However, question has been raised as to the accuracy of the former method of testing. The results of this study demonstrate that there is poor agreement between the values obtained by the disc and tube dilution methods with certain antibiotics. It is therefore felt that there is a sacrifice of accuracy for simplicity of testing when the disc method of sensitivity determination is used.

There was good agreement between the results of the agar dilution and tube dilution methods of testing. The fact that the antibiotic-containing plates can be kept in the refrigerator over a period of 4 weeks (3 weeks in the case of aureomycin) without loss of potency means that series of plates can be prepared once every 2 to 3 weeks and stored until needed for testing. There are several points that are important in the preparation of antibiotic-containing plates. It is important, especially with aureomycin, to avoid letting solutions of antibiotics stand before incorporating them in plates because some decrease in potency occurs(7,9). Of prime importance in the preparation of such plates is the cooling of the agar to 45°C before adding an antibiotic; this must be observed in order to prevent heat destruction of the antibiotic. Plates to be stored must be kept in a refrigerator at a temperature of 4°C.

In these experiments the size of the inoculum used did not alter the results of the agar dilution method. These findings are in agreement with those of others(10-12).

The agar dilution method of testing is simple in its performance and its accuracy agrees with that of the tube dilution method within limits of clinical usefulness. Like the latter method, the agar dilution method offers a direct estimate of the concentration of antibiotic necessary for inhibition of an organism.

Conclusions. 1. The agar dilution and tube dilution methods of determining bacterial sensitivity are equally reliable. 2. Aureomycin-containing plates can be stored for 3 weeks without loss of potency; plates containing penicillin, bacitracin, streptomycin, chloramphenicol, and terramycin can be stored for 4 weeks without loss of potency. 3. The agar diffusion method of sensitivity determination is not fully reliable, as shown by poor correlation of the results obtained by that method and the tube dilution method of testing with some antibiotics.

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Biliary and Urinary I^{131} Excretion in Euthyroid, Hyper- and Hypothyroid Rats Injected with Labeled Thyroxine. (20184)

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Reports in the literature indicate that thyroxine or its metabolites are excreted in the bile and urine of normal animals(1). The object of the present study is to determine to what extent this elimination is altered in hyper- and hypothyroid animals. The effects of desiccated thyroid treatment, thyroidectomy, and thiouracil administration on the excretion of I^{131} were studied in rats following the injection of I^{131}-labeled thyroxine.

Methods and material. Adult male rats of the Sprague-Dawley strain were used. Normal and thyroidectomized groups were maintained on Rockland Complete Rat Diet. Thyroidectomies were performed under ether anesthesia, with at least 6 weeks allowed for recovery and development of hypometabolism (2). The third group received the same diet supplemented with 2 g of thiouracil per kg of diet for 4 weeks before being used(2). The fourth group was maintained on the basic diet containing 0.2% desiccated thyroid for 3 weeks to allow time for development of marked hypermetabolism. All animals were fed *ad lib.* Two lots of I^{131}-labeled thyroxine (Abbott)* having specific activities of 8.1 and 35.4 mc per mg, respectively, were used. Tracer amounts of about 0.2 μg per kg of body weight were administered subcutaneously. In the first series of experiments, the bile duct was cannulated with polyethylene tubing (inside diameter, 0.011 inch) while the rat was under ether anesthesia. As soon as bile flow was observed, the abdomen was closed, and the animal suspended in a restraining harness. The tracer amount of labeled thyroxine was injected subcutaneously and hourly bile samples were collected. Radioactivity of the whole bile was determined by air drying 0.1 ml aliquots on aluminum plates, and counting in a thin mica window Geiger counter. Urine was collected from a second series of animals placed in metabolic cages. The concentration of I^{131} activity in the urine was determined on 0.5 ml samples and counted in the manner described for bile.

TABLE I. Cumulative 6-Hour Biliary Excretion Following Subcutaneous Injection of I^{131}-Labeled Thyroxine. 4 rats in each series.

Condition	% activity excreted ($x \pm s_x$)*	P†	ml bile excreted*	P†
Normal	11.6±3.08	—	6.1±1.23	—
Desiccated thyroid-treated	12.3±2.38	.703	8.5±1.83	.070
Thyroidectomized	6.2±1.34	.019	3.1±0.78	.006
Thiouracil-treated	3.2±3.02	.009	3.5±2.02	.070

* x = sample mean; s_x = stand. dev. of values.
† Probability of no difference from normal.

Results and discussion. Results with biliary excretion are shown in Table I. Mean excretion of radioiodine in whole bile of normal animals amounted to 11.6% of the injected dose by 6 hours after administration. Taurog and co-workers(3) give a comparable value of about 13% excretion after a subcutaneous injection of 100 μg per kg of I^{131}-labeled L-thyroxine during the same period of time. The radioactivity eliminated in the thyroidectomized and thiouracil-treated rats was reduced significantly to 6.2 and 3.2%, respectively. Desiccated thyroid treatment did not alter significantly the per cent of injected activity excreted in the bile. During the 6-hour collection period the euthyroid animals eliminated 6.1 ml of bile while the excreted volume was reduced to 3.1 and 3.5 ml in the thyroidectomized and thiouracil-treated groups, respectively. An increase to 8.5 ml was found in hyperthyroid rats.

As indicated in Table I, the per cent of injected activity excreted in the bile was measured in 4 thiouracil-treated rats. One animal in this group, however, was more than 3 standard deviations from the mean of the

* The I^{131} used in this investigation was obtained from the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

TABLE II. Cumulative Twelve-Hour Urinary Excretion Following Subcutaneous Injection of I^{131}-Labeled Thyroxine.

Condition	No. of rats	% activity excreted ($x \pm s_x$)*	P†
Normal	4	1.3 ± 0.22	—
Desiccated thyroid-treated	4	3.8 ± 1.73	.031
Thyroidectomized	7	1.8 ± 0.79	.261
Thiouracil-treated	4	0.9 ± 0.26	.053

* x = sample mean; s_x = stand. dev. of values.
† Probability of no difference from normal.

remaining animals. Because of the small number of animals used it has been included in the table. By excluding this animal from the group, the mean per cent of I^{131} excreted in the bile becomes 1.7. This mean value is markedly less ($P = .003$) than the corresponding value in the thyroidectomized group. If the apparently atypical animal is included the difference is not highly significant ($P = 0.12$). In comparing the desiccated thyroid-treated group with that of the normal, the former showed an increase in the volume of bile eliminated with no alteration in the per cent of radioactive iodine excreted. The elimination of radioiodine is not related to the amount of bile excreted. However, the volume of bile does appear to be directly related to the thyroid status.

The results of urinary excretion are shown in Table II. Excretion of radioactive iodine in the urine of euthyroid rats amounted to 1.3% of the injected dose of thyroxine by 12 hours after administration. The quantity appearing in the urine of hyperthyroid and thyroidectomized groups was increased to 3.8 and 1.8%, respectively. A decrease to 0.9% was found in the thiouracil-treated rats. Reports in the literature indicate that the kidney plays an important part in the excretion of iodine, but not in the excretion of thyroxine *per se* (1). The increased rates may be due to the marked increase in metabolism which in turn would result in an increase in available radioiodine containing metabolites from the administered thyroxine.

A trend toward a lower rate of excretion in thiouracil animals with respect to thyroidectomized animals is seen in Table II ($P = 0.06$). These results suggest that thiouracil *per se* has an effect on the urinary and biliary excretion of radioiodine in the rat. The ability of the thyroid gland to concentrate iodide is greatly increased by thiouracil treatment(4). In the present series of experimental treatments this might explain the order of urinary radioiodine excretion found among the normal and hypothyroid groups. This does not explain the corresponding values for biliary excretion. Recent investigations(5,6) indicate that thyroxine is eliminated in bile in a free and/or conjugated form. The lowered metabolism of the thyroidectomized and thiouracil-treated groups may be the basis for lower than normal biliary excretion of I^{131}, but the reason for the difference between the 2 hypometabolic groups is not clear. Toxic effects of thiouracil might explain the results found.

Summary. Biliary and urinary excretion of radioiodine was studied following subcutaneous injection of tracer amounts of I^{131}-labeled thyroxine in normal, hypo- and hyperthyroid rats. Thyroidectomized and thiouracil-treated groups showed reduced biliary radioiodine elimination, as well as diminished bile volume during the 6-hour collection period. Hyperthyroid animals showed a marked increase, thiouracil-treated animals a decrease in urinary excretion of radioactive iodine over a 12-hour period. Radioiodine excretion in both bile and urine tended to be lower in the thiouracil-treated animals than in the thyroidectomized groups.

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Augmentation of Nitrogen Mustard Induced Leukopenia by Cortisone.* (20185)

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The administration of L-cysteine prior to the injection of nitrogen mustard (HN_2) results in a decrease in the degree of leukopenia characteristically induced by HN_2(1,2). This protective effect is dependent upon the presence within the molecule of a free sulfhydryl, amino and carboxyl group in close apposition(3). Thus, compounds with structures closely related to L-cysteine are ineffective in preventing leukopenia if this specific configuration is altered. This protective effect of L-cysteine is apparently not due to chemical inactivation of HN_2, and may be due to the protection of some substance or substances essential for leukopoiesis from destruction by HN_2. However, the possibility exists that L-cysteine may also protect tumor tissue as well as hematopoietic tissue against the effects of HN_2. Consequently, other compounds which could protect hematopoietic tissue without the possibility of interfering with the beneficial effects of HN_2 would be desirable.

ACTH and cortisone are known to produce granulopoiesis(4,5), and it is therefore possible that these compounds might be beneficial against HN_2-induced leukopenia. Furthermore, ACTH and cortisone are frequently therapeutically effective in the same diseases as HN_2, and it has been suggested that combined therapy with these 2 agents might be beneficial. Accordingly, the effect of cortisone on HN_2-induced leukopenia was investigated in rabbits. In addition, the effect of cortisone in modifying the L-cysteine protection of HN_2-induced leukopenia was also studied.

Materials and methods. The effect of cortisone on the leukopenia induced by HN_2 was studied by comparing the maximum fall in total leukocyte count and polymorphonuclear leukocyte count in rabbits receiving HN_2 alone and in rabbits receiving cortisone either before or after HN_2 administration. HN_2 [methyl bis (beta-chlorethyl) amine hydrochloride]‡ was administered intravenously in a single dose of 2.5 mg per kg body weight in all experiments. This dose invariably induces a maximum leukopenia on the third to sixth day after injection. Cortisone§ was administered intramuscularly in doses of 5 mg per kg body weight either twice daily for 2 days before HN_2 administration or once daily for 3 days following HN_2 administration (Table I). Control studies were obtained by giving intramuscular injections of either desoxycorticosterone acetate (DOCA)‖ or normal saline in place of cortisone. DOCA was administered in doses of 1.0 mg per kg body weight daily for 2 days before or for 3 days following HN_2 administration. Normal saline was given in 1.0 cc doses daily for 3 days following HN_2 (Table I). The effect of cortisone on the protective effect of L-cysteine against HN_2-induced leukopenia was investigated by giving cortisone to rabbits either before or after combined L-cysteine-HN_2 therapy. L-cysteine hydrochloride was prepared as a 20% solution(1) and administered intravenously in doses of 500 mg per kg body weight immediately before HN_2 injection. Cortisone was given in doses of 5.0 mg per kg body weight intramuscularly twice daily for 2 days before, or once daily for 3 days after combined L-cysteine-HN_2 therapy (Table II). Control studies using DOCA instead of cortisone were done. DOCA was given in doses of

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‡ Nitrogen mustard and L-cysteine hydrochloride were furnished through the courtesy of Merck and Co., Rahway, N. J.

§ Saline suspension of cortisone acetate (Merck), 25 mg per cc.

‖ Desoxycorticosterone acetate in sesame oil, 5 mg per cc.

TABLE I. Effect of Cortisone on Nitrogen Mustard (HN_2)-Induced Leukopenia.

Treatment	No. of rabbits	Max fall in WBC's* (% of control count)	Max fall in PMNS* (% of control count)	Lymphopenia, (during max leukopenia) (% of control count)
Nitrogen mustard (NH_2) 2.5 mg/kg I.V.	9	82	93	77
Cortisone 5 mg/kg I.M. b.i.d. × 2 before HN_2	10	91†	96†	87†
Cortisone 5 mg/kg I.M. q.d. × 3 after HN_2	10			
DOCA 1.0 mg/kg I.M. q.d. × 2 before HN_2	9	88‡	97‡	83‡
DOCA 1.0 mg/kg I.M. q.d. × 3 after HN_2	10			
Saline 1.0 cc I.M. q.d. × 3 after HN_2	10	81	94	74
Sham operation immediately before HN_2	7	95	94	94

* Max fall $= \frac{\text{Initial count} - \text{lowest count}}{\text{Initial count}} \times 100.$

† Results essentially the same whether cortisone is given before or after HN_2 administration.
‡ Administration of DOCA either before or after HN_2 produces essentially same results.

1.0 mg per kg body weight intramuscularly daily for 2 days before or daily for 3 days after combined L-cysteine-HN_2 therapy (Table II). Total leukocyte and differential counts were done daily from the beginning of the experiment until recovery from the maximum leukopenia was evident.

Results. Administration of HN_2 alone resulted in an average maximum fall in total leukocyte count of 82% of control counts and an average maximum fall of neutrophils of 93% of control counts. The absolute lymphocyte count fell 77% of the control counts. Administration of cortisone either before or after HN_2 injection increased the average maximum fall in total leukocyte count to 91% (*i.e.* 9% more than with HN_2 alone) and the total lymphocyte count to 87% (10% more than with HN_2 alone), without any significant increase in the degree of neutropenia. In the rabbits receiving DOCA, in place of cortisone, the average maximum fall in total leukocyte, neutrophil and lymphocyte counts was 88, 97 and 83%, respectively. Administration of normal saline intramuscularly following HN injection caused no significant change in total leukocyte counts or differential counts compared to animals receiving HN_2 alone (Table I).

Rabbits given L-cysteine hydrochloride immediately prior to HN_2 administration showed an average maximum fall in total leukocyte count of only 37% of initial counts, while the average maximum fall in absolute neutrophil and lymphocyte count was only 32 and 45%, respectively (Table II). When cortisone was administered either before or after combined L-cysteine-HN_2 injections, the average maximum fall in total leukocyte count increased to 64% (27% more than with L-cysteine and HN_2 alone), and the fall in absolute neutrophil and lymphocyte counts increased to 59 and 73%, respectively (27 and 28% more than with L-cysteine and HN_2 alone). Control animals receiving DOCA instead of cortisone showed average maximum falls in total leukocyte, neutrophil and lymphocyte counts of 46, 51 and 50%, respectively (Table II).

Discussion. Although cortisone usually causes a leukocytosis in animals and in humans, it is apparent that under the conditions of this experiment, cortisone offered no protection against the leukopenia induced by HN_2 in rabbits. The administration of cortisone caused an even greater drop in total leukocyte count than in animals receiving HN_2 alone. The greater total leukopenia resulting from cortisone administration is due to an increased lymphopenia.

Endogenously produced ACTH and corti-

TABLE II. Effect of Cortisone in Modifying Protective Effect of L-Cysteine on Nitrogen Mustard (HN_2)-Induced Leukopenia.

Treatment	No. of rabbits	Max fall in WBC's* (% of control count)	Max fall in PMNS* (% of control count)	Lymphopenia, (during max leukopenia) (% of control count)
L-Cysteine 500 mg/kg I.V. immediately before HN_2 2.5 mg/kg I.V.	16	37	32	45
Cortisone 5.0 mg/kg I.M. b.i.d. × 2 before L-Cysteine and HN_2	10	64†	59†	73†
Cortisone 5.0 mg/kg I.M. q.d. × 3 after L-Cysteine and HN_2	9			
DOCA 1.0 mg/kg I.M. q.d. × 2 before L-Cysteine and HN_2	9	46‡	51‡	50‡
DOCA 1.0 mg/kg I.M. q.d. × 3 after L-Cysteine and HN_2	9			

* Max fall $= \frac{\text{Initial count} - \text{lowest count}}{\text{Initial count}} \times 100.$

† Results essentially the same whether cortisone is given before or after HN_2 administration.

‡ Administration of DOCA either before or after HN_2 produces essentially same results.

sone apparently have the same effect on HN_2-induced leukopenia as cortisone administered parenterally. Thus, when rabbits were subjected to either a splenectomy or to a sham operation, consisting solely of an abdominal incision, immediately prior to HN_2 administration, a marked increase in the degree of leukopenia was noted compared to animals receiving the same amount of HN_2 per kg body weight without an operation. In these animals, the increased leukopenia was also due to a greater lymphopenia (Table I).

Cortisone likewise failed to augment the protective effect of L-cysteine on HN_2-induced leukopenia. On the contrary, administration of cortisone to rabbits receiving L-cysteine immediately prior to HN_2 injection resulted in a greater degree of leukopenia, neutropenia, and lymphopenia than in animals receiving L-cysteine and HN_2 alone. Cortisone, therefore, appears either to diminish the protective effect of L-cysteine or to augment the effect of HN_2 in producing leukopenia.

The administration of DOCA with HN_2 or with combined L-cysteine-HN injections also augments the resulting leukopenia. However, the effects of DOCA are not as marked as with the administration of cortisone.

Graham *et al.*(6) have found that cortisone administered immediately following irradiation in mice had no effect on the survival rate of these animals. Thiersch *et al.*(7) administered cortisone to rats daily for 2 weeks beginning on the third day following whole body radiation. They found that cortisone administered after irradiation damage was established, caused a greater depression of bone marrow and a slower rate of recovery than in animals receiving x-ray alone. On the other hand, Mirand *et al.*(8) demonstrated that both cortisone and DOCA given to mice either before or immediately after irradiation afforded protection from the lethal effects of ionizing radiation. The failure of cortisone to prevent HN_2-induced leukopenia in our studies is similar to the results of Graham and Thiersch in which cortisone failed to favorably influence the results of irradiation.

These findings indicate that no benefit can be expected from the combined use of cortisone and HN_2 as far as protection of leukocytes is concerned. Although treatment of certain diseases with both cortisone and HN_2 may be therapeutically synergistic, combined therapy must be considered dangerous from the standpoint of the severe depression of hematopoietic tissue which may result.

Summary. 1. Cortisone is ineffective in pre-

venting HN_2-induced leukopenia in rabbits. On the contrary, cortisone induces a greater leukopenia as a result of increased lymphopenia. 2. Administration of cortisone to rabbits receiving L-cysteine prior to HN_2 administration results in a more severe leukopenia and neutropenia than administration of L-cysteine and HN_2 alone. 3. The combined use of cortisone and HN_2 therapeutically may induce a dangerous depression of hematopoietic tissue.

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Growth-Promotion by Lyxoflavin. II. Relationship to Riboflavin in Bacteria and Chicks.* (20186)

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Addition of L-lyxoflavin to appropriate experimental rations increases growth of rats (1-3), chicks(3,4), and pigs(5). To explain this action it has been suggested(2,4) that lyxoflavin may have vitamin action of its own, since: (a) it has no growth-promoting action for higher animals in the absence of riboflavin, (b) its addition to diets similar to those used for assay of unidentified factors stimulates growth, and (c) it has been reported to occur naturally(6), implicating a functional role for it in metabolism. The merit of these arguments is open to question. Growth stimulation by a given substance does not prove vitamin activity, as is abundantly evident from the growth-promoting action of a variety of antibiotics. Furthermore, although lyxoflavin stimulates growth of chicks on rations used for assay of unidentified stimulants, addition of crude supplements known to contain adequate amounts of these unidentified factors did not mask the response to lyxoflavin(4) as would be expected if their growth-promoting activity was due to this substance. Finally, the evidence for the natural occurrence of lyxoflavin is inconclusive(7); unless the compound actually occurs naturally, vitamin activity can scarcely be ascribed to it.

These findings with experimental animals are superficially similar to those in bacteria. We reported previously, without confirming data, that although lyxoflavin did not permit growth of *Lactobacillus casei* in the absence of riboflavin, low concentrations of lyxoflavin did increase the growth response of this organism to limiting amounts of riboflavin(4). Lyxoflavin resembled in this respect D- and L-araboflavins(8). At higher concentrations, lyxoflavin inhibits growth by acting as a competitive antagonist of riboflavin(4). These findings, which have been confirmed by others (3,9) are extended below. The report of Shorb(9) that *Lactobacillus lactis* grows in the absence of riboflavin when lyxoflavin is supplied has been confirmed and extended.

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Finally, it is shown that lyxoflavin, at high concentrations, acts as a riboflavin antagonist in chicks. Many toxic compounds stimulate growth in subinhibitory concentrations (10,11) and, although no generally applicable explanation for this behavior is known, it appears possible that stimulation of animal growth by lyxoflavin may represent an instance of this same phenomenon.

Procedures. a. *Bacterial experiments.* The assay medium used with *L. casei* 7469 was that described by Rabinowitz *et al.* (12) modified by omission of riboflavin and additon of 80 γ of pyridoxine hydrochloride and 0.1 mg each of adenine, guanine, and uracil for each 10 ml. For *L. lactis* 8000 this basal medium was supplemented with 5 mg of sodium ethyl oxaloacetate, 10 mg of polyoxyethylene sorbitan monopalmitate (Tween 40), 0.1 mg of sodium oleate, 10 γ of calcium pyridoxal phosphate, 2.5 γ of KCN, 0.04 γ of vit. B_{12}, and 0.025 γ of pantethine per 10 ml. Inoculum cells were grown for 18-24 hours in the appropriate basal medium supplemented with 1 γ of riboflavin per 10 ml, centrifuged, resuspended in sterile 0.9% NaCl solution, and diluted to a light transmission of 83% (Evelyn colorimeter, 660 mμ filter). One drop of this suspension was used for each experimental culture of 10 ml. Assays were incubated at 37°C; growth was determined turbidimetrically at appropriate times indicated with individual experiments. b. *Chick experiments.* Procedures were similar to those described previously (4). Day-old chicks (NH x SCWL) were separated into groups of 10 to 15 birds. They were maintained in electrically heated batteries and fed the rations and water *ad lib.* The basal ration was as follows, in g/kg: sucrose 630, alpha protein (Glidden) 250, salts V 60, soybean oil 45, feeding oil (300D-1500A) 5, DL-methionine 6, and glycine 3. Vitamin supplements in mg/kg were: vit. B_{12} 0.02, biotin 0.2, menadione 0.5, folacin 4, pyridoxine hydrochloride 4, calcium pantothenate 20, α-tocopherol acetate 3, niacin 50, p-aminobenzoic acid 100, inositol 1000, choline chloride 2000, and aureomycin 20. Thiamine was supplied by injection thrice weekly at levels of 0.5 mg per chick per injection the first week, and 1.0 mg during the second week. The experimental period was 15 days. Riboflavin and its analogs were fed at levels noted in the individual experiments.

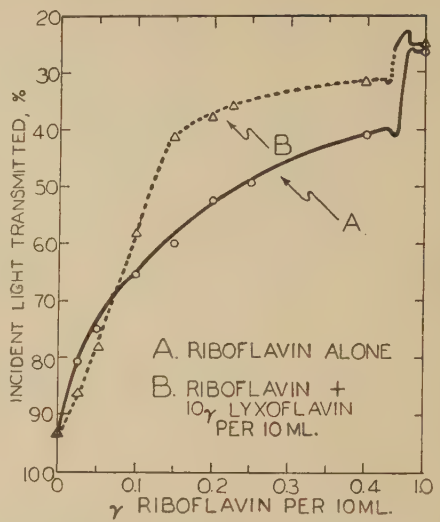

FIG. 1. Effect of a constant low level of L-lyxoflavin on response of *L. casei* to riboflavin. Incubated 17 hr.

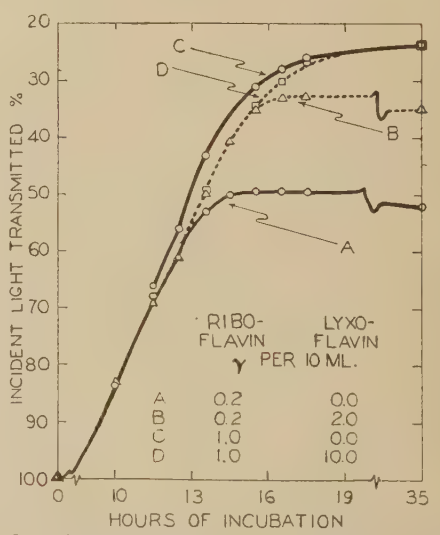

FIG. 2. Effect of riboflavin or riboflavin plus L-lyxoflavin on rate and extent of growth of *L. casei*.

Results. a. *Bacterial experiments.* The effect of a constant low level of L-lyxoflavin on the response of *L. casei* to riboflavin is shown in Fig. 1. At ratios of lyxoflavin to riboflavin above 100 to 1, inhibition of the response to riboflavin occurs; at ratios lower than this, stimulation of the response is ob-

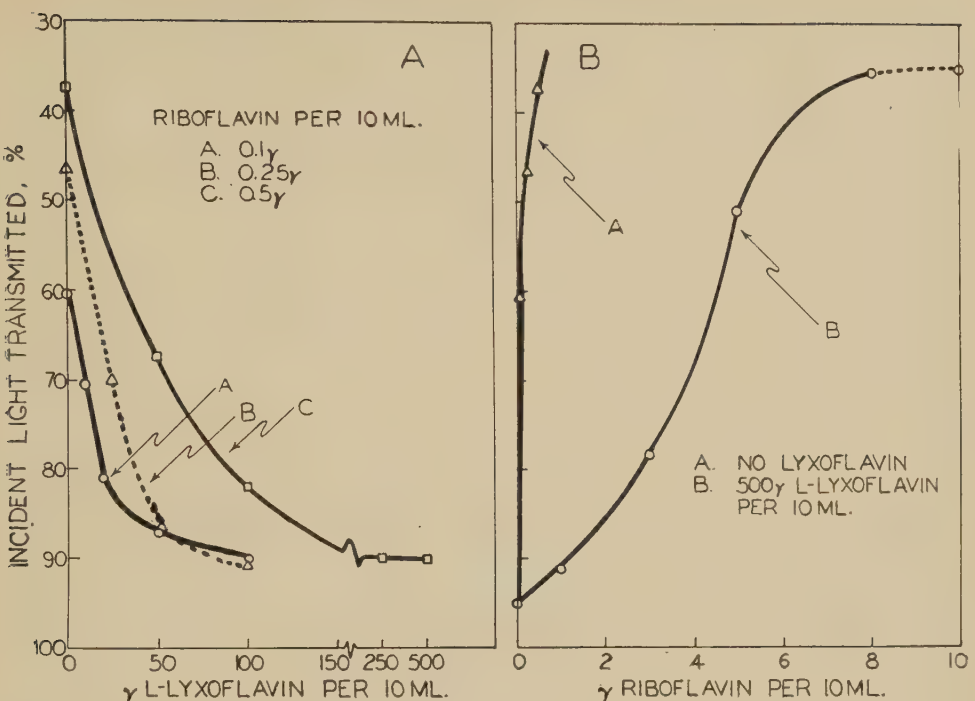

FIG. 3. Inhibition of response of *L. casei* to riboflavin by high levels of L-lyxoflavin. Incubated 17 hr.

served. With sufficient riboflavin, growth reaches the same maximum levels achieved with riboflavin plus lyxoflavin. In the absence of riboflavin, lyxoflavin permits no growth of *L. casei* (Fig. 1).

Data of Fig. 2 show that these levels of lyxoflavin act solely to increase the *amount* of growth when riboflavin is limiting; there is no stimulation of the rate of growth.

That inhibition by high levels of lyxoflavin is competitively related to the level of riboflavin present is evident from Fig. 3. Growth with 0.05 γ of riboflavin gives a light transmission of 70. When the ratio of lyxoflavin to riboflavin required to reduce growth to this level is calculated from the 4 curves of Fig. 3, values of 190, 125, 123, and 133 are obtained.‡ Very high levels of lyxoflavin are non-toxic if sufficient riboflavin is present (Fig. 3B).

‡ The ratios, R, were calculated according to the expression $R = \frac{C_L}{C_R - 0.05}$, where C_L is the amount of lyxoflavin (in γ) and C_R that of riboflavin present at growth equivalent to 70% light transmission.

The results shown in Fig. 4 emphasize the pronounced growth stimulation produced by subinhibitory amounts of L-lyxoflavin when riboflavin is limiting. D-galactoflavin behaves in the same way, but is a less potent inhibitor. Separate tests showed that inhibition by galactoflavin, like that by lyxoflavin, is competitively related to the concentration of riboflavin. The inhibitory action of this compound for *L. casei* correlates with its action in rats(13); growth stimulation in animals by subinhibitory levels of the compound has not been noted. Isoriboflavin showed the growth stimulation characteristic of the other 2 compounds, but in confirmation of a previous report(14) did not inhibit growth at levels which could be tested. The compound is an antimetabolite of riboflavin in rats(15). Dichlororiboflavin, in contrast to its action in several other bacteria(16), was largely inert toward *L. casei*.

In contrast to a report of Shorb(9), no essential change in these results was observed when the riboflavin-free medium of Snell and Strong was substituted for the more nearly

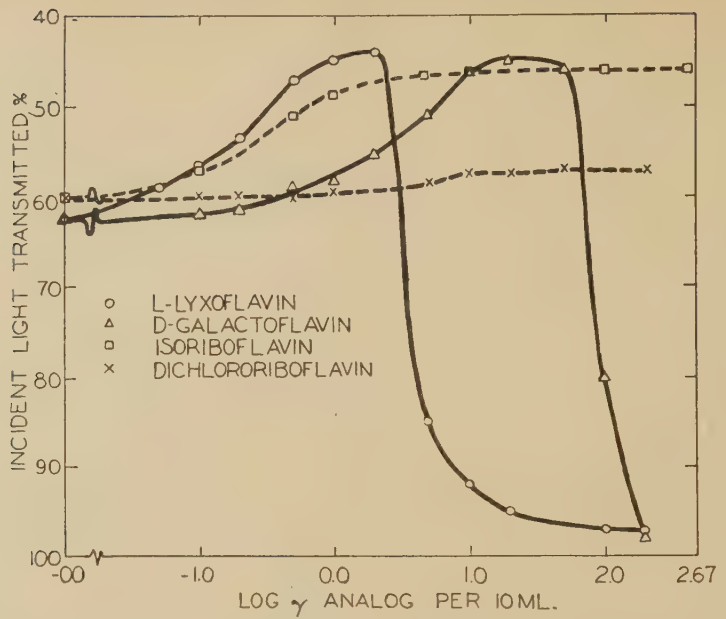

FIG. 4. Comparative effects of several analogs of riboflavin on growth of *L. casei*. Each culture tube was supplemented with 0.1 γ of riboflavin per 10 ml. None of the analogs permits growth in the absence of riboflavin.

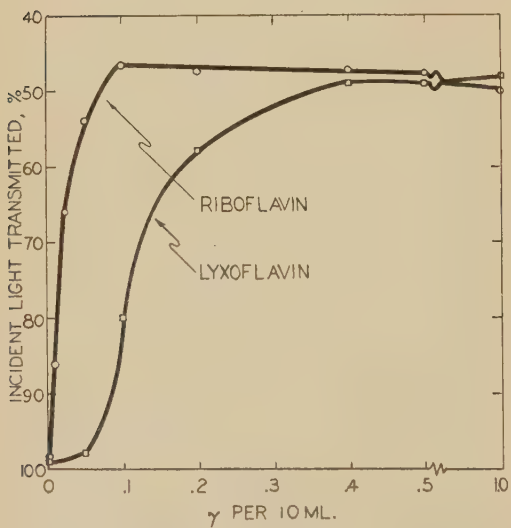

FIG. 5. Comparative effects of riboflavin and L-lyxoflavin on growth of *L. lactis*. Incubated 38 hr.

synthetic medium used in most of the experiments.

L. lactis behaves differently than *L. casei*. As first reported by Shorb(9), L-lyxoflavin supports as heavy growth of the former organism as does riboflavin, but is only one-tenth to one-third as active on a molar basis (Fig. 5). Growth of *L. lactis* with L-lyxoflavin as the sole supplement to the riboflavin-free medium continued without diminution through 7 consecutive transfers; there is thus no doubt of its ability to support growth of this organism in the absence of added riboflavin. Galactoflavin and isoriboflavin did not permit growth of *L. lactis* in the absence of riboflavin.

To determine whether lyxoflavin was used *per se* by *L. lactis*, or was converted to riboflavin, cells of this organism grown with riboflavin or lyxoflavin were harvested, washed once with water, then autoclaved for 20 minutes at 15 lb with 0.1 N HCl to release bound flavins. The extracts were then assayed in parallel with *L. casei* and *L. lactis*, against a riboflavin standard, and in the basal medium for *L. lactis*. Results (Table I) show that cells grown with riboflavin contained the same amount of flavin, within experimental error, by both assay methods. Cells grown with lyxoflavin contained no riboflavin (*L. casei* assay), but did contain lyxoflavin. These data and those of Fig. 5 indicate conclusively that lyxoflavin replaces riboflavin in the metabolism of *L. lactis*, presumably by incorporation into functional lyxoflavin coenzymes

TABLE I. Flavin Content of *L. lactis* Cells Grown with Riboflavin or Lyxoflavin.

Cells grown with	Flavin content,* γ/mg of cells, determined with *L. casei*†	*L. lactis*‡
Riboflavin (.01 γ/ml)	.073	.085
L-lyxoflavin (.03 γ/ml)	.00	.076§

* Riboflavin as standard.

† Specific for riboflavin.

‡ Responds to riboflavin and to lyxoflavin, see text.

§ Since lyxoflavin is only about one-third as active as riboflavin in promoting growth, this would correspond to a lyxoflavin content of approximately 0.23 γ/mg of cells.

TABLE II. Comparative Flavin Content of Cells of *L. casei* Grown with Riboflavin Alone vs. Riboflavin plus Stimulatory Levels of L-Lyxoflavin.

Cells grown with— Riboflavin, γ/250 ml	L-lyxoflavin, γ/250 ml	Yield of dry cells, mg	Total flavin content by *L. lactis* assay* γ/mg of cells	Total γ
2.5	0	27.8	.033	.92
2.5	25	48	.022	1.03
2.5	250	37	.017	.64
25.0	0	84	.17	14.2

* Assayed against a riboflavin standard and expressed as riboflavin. Similar results, not shown, were obtained with *L. casei*, which does not respond to lyxoflavin.

analogous to those normally formed from riboflavin.

A similar experiment should reveal whether the sparing effect of lyxoflavin on the riboflavin requirement of *L. casei* results from utilization of lyxoflavin for some but not all of the purposes normally served by riboflavin. Accordingly cells of *L. casei* were grown with a suboptimal level of riboflavin, or with this level plus a stimulatory amount of lyxoflavin, and assayed for lyxoflavin plus riboflavin with *L. lactis*. Results (Table II) showed that the 48 mg of cells obtained from a medium containing suboptimal riboflavin plus lyxoflavin contained no more total flavin than the 28 mg of cells obtained with riboflavin alone. There is thus no evidence for the incorporaiton of lyxoflavin into these cells, a conclusion borne out by parallel assays conducted with *L. casei*. The concentration of riboflavin per mg of cells is decreased by culture in the presence of lyxoflavin; *i.e.*, the incorporation of riboflavin appears to be decreased by concentrations of lyxoflavin that are stimulatory. Increasing levels of lyxoflavin progressively decrease the amount of riboflavin incorporated in the cells; presumably growth inhibition results when this incorporation is decreased below a necessary minimum value. Thus lyxoflavin appears to "spare" riboflavin in *L. casei* not by functioning in its stead, but rather by causing (by means still unknown) a more efficient utilization of the limited amounts of riboflavin available. That riboflavin may be deposited in cells at concentrations far higher than are required for growth is evident from the high level present in those cells grown with an excess of this vitamin (Table II).

When this same differential assay procedure was applied to heart, brain, liver, kidney and leg muscle of adult rats grown on a stock diet based on crude natural foodstuffs (Purina Laboratory Chow), the flavin content was the same within experimental error whether *L. casei* or *L. lactis* was the assay organism. This is strong evidence against the natural occurrence of lyxoflavin.

b. *Chick experiments.* Results of feeding L-lyxoflavin and riboflavin at several different levels are shown in Fig. 6. Growth stimulation is consistently observed when ratios of lyxoflavin to riboflavin are 10:1 or below. At considerably higher levels lyxoflavin inhibits growth; the amount necessary for inhibition increases as the amount of riboflavin in the ration is increased. The relationship thus appears to be of the competitive type demonstrated earlier in *L. casei*. Cooperman *et al.* (3) suggested that further additions of riboflavin, above 6 mg/kg, stimulated an increase in growth similar to that elicited by lyxoflavin. Under our conditions, 3 mg of riboflavin per kg promotes the maximum growth obtainable with riboflavin alone. Although, on a percentage basis, lyxoflavin increases growth somewhat more at suboptimal levels of riboflavin, growth at superoptimal levels of riboflavin is also increased by additions of lyxoflavin (Table III). It is unlikely, therefore, that lyxoflavin acts *solely* by "sparing" riboflavin in the chick. These data suggest that the amount of lyxoflavin required to stimulate growth, like that required to inhibit

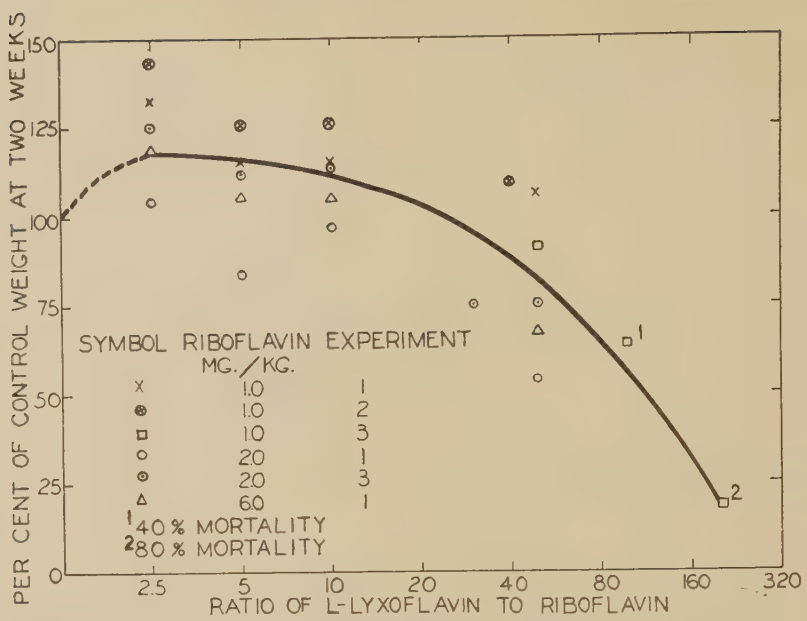

FIG. 6. Effect of lyxoflavin on growth of chicks fed various levels of riboflavin. The curve was fitted by the method of least squares, $Y = 119 - 0.824X + 0.00164X^2$.

growth, increases as the riboflavin content of the ration is increased, *i.e.*, maximum growth increases are obtained in all cases at a ratio of 2.5:1. However, since lower levels of lyxoflavin have not been adequately tested, data on this point are inconclusive.

With the possible exception of D-lyxoflavin, the other analogs of riboflavin were neither stimulatory nor inhibitory for chicks when fed with the maintenance level (1 mg/kg) of riboflavin (Table IV). The small amounts of these chemicals available made trials at higher levels impossible. From these results, galactoflavin would appear less potent as an antagonist for riboflavin in chicks than in rats.

TABLE III. Effect of Lyxoflavin on Chick Growth at Superoptimal Levels of Riboflavin. Gain in 14 days, g.

	mg riboflavin/kg ration				
	0	3	6	12	24
No lyxoflavin	5	72	71	73	71
L-lyxoflavin: riboflavin (2.5:1)	—	—	72	80	80

TABLE IV. Comparative Effects of Several Riboflavin Analogs on Chick Growth.* Avg wt of chicks at 15 days.

Analog fed	mg analog/kg ration					
	0	2.5	5.0	10	20	40
L-lyxoflavin	60	70	66	66		62
D-lyxoflavin	60	66	64	62	57	55
D-araboflavin	60	61	63	55	57	57
D-galactoflavin	60	60	60	60	58	61

* 1 mg riboflavin/kg of ration.

Discussion. The demonstration that lyxoflavin replaces riboflavin completely in the nutrition of *L. lactis,* and functions in this organism without prior conversion to riboflavin adds another instance to several of this type already known. Oxybiotin, although frequently less active quantitatively, effectively replaces biotin in the nutrition of many microorganisms and animals, and is not converted to biotin(11,17,18). No cases of inhibition of growth by oxybiotin are known. A closer analogy is provided by Rb^+, which replaces K^+ completely for *Streptococcus faecalis,* but not for *Leuconostoc mesenteroides.* At low levels it greatly reduces the requirement of the latter organism for K^+, apparently serving in its stead in some but not all metabolic reactions. At high levels, Rb^+ inhibits growth by competition with K^+ at sites where the latter is essential for growth(19,20).

Another probable instance of this same phenomenon is provided by analogs of riboflavin appropriately substituted in the 6- and

7- positions of the alloxazine nucleus, some of which have activity for microorganisms(8,11) and partial activity in animals(11). The most thoroughly examined of these is "diethylriboflavin"(21), which replaces riboflavin efficiently and completely for *L. casei* and *Bacillus lactis acidi,* and which stimulates growth of rats on riboflavin-deficient diets, but does not prevent their death when deprivation of riboflavin is continued. Although the growth effect of the analog in the latter instance was ascribed(2) to a mobilizing effect on riboflavin rather than to utilization *per se,* the possibility that it replaced riboflavin for certain functions but not for others was not eliminated. It appears unlikely from chemical considerations that these analogs are transformed to riboflavin by organisms that utilize them, although the experiments necessary to prove this have not been carried out.

An attractive postulate to explain the effects of lyxoflavin on growth of *L. casei* would be to assume that this compound could fill some but not all of the necessary functions of riboflavin. At subinhibitory levels it would then stimulate growth when riboflavin was limiting; at higher concentrations, by interfering with essential functions of riboflavin, it would inhibit growth. Evidence of Table II indicates, however, that lyxoflavin does not partially substitute for riboflavin, but rather "spares" riboflavin by interfering with its deposition at non-essential sites within the cell. At higher, growth-inhibitory concentrations, deposition of riboflavin at essential sites also must be prevented. The important point is that growth-stimulation at low concentrations of lyxoflavin thus results from operation of the same mechanism that causes growth inhibition at high concentrations. In the same way, other riboflavin antagonists might show a similar stimulating effect for this organism at sub-inhibitory concentrations; this expectation is fulfilled by D-galactoflavin. Growth stimulation without subsequent growth inhibition by isoriboflavin is not contradictory to this view, since an essential feature of the hypothesis is that deposition of riboflavin at non-essential sites is more readily inhibited than that at essential sites, *i.e.,* the cell receptors at non-essential sites have a lower "affinity" for riboflavin or its coenzyme forms than do cell-receptors at the essential sites.

In theory, riboflavin antagonists might also exist with specificity such that riboflavin would be prevented from combination with essential cell-receptors at concentrations as low or lower than that required to prevent combination with non-essential receptors. In such case, stimulation of growth by subinhibitory concentrations of the antagonist would not be observed. None of the riboflavin antagonists tested acted in this manner.

From Fig. 1 and 4 it is apparent that under conditions where it is maximally effective, lyxoflavin possesses only a small fraction of the activity of riboflavin in promoting growth of *L. casei.* Chemically, however, lyxoflavin and riboflavin are very similar. Since results of microbiological assays for riboflavin employing *L. casei* have been checked extensively against fluorometric assay with excellent agreement(22,23), and since such agreement would be impossible if lyxoflavin were widely distributed in nature in amounts comparable to riboflavin, it must be concluded that lyxoflavin, if it occurs naturally at all, must be present in very small amounts relative to riboflavin. This conclusion is supported by the agreement between results of assay of natural materials with *L. casei* and *L. lactis.* If lyxoflavin occurred naturally, the latter organism should give higher assay values; in trials on rat tissues it did not.

The growth responses to lyxoflavin observed in chicks on diets marginal in riboflavin may be due in part to operation of factors similar to those discussed for bacteria. In contrast to the results in *L. casei,* however, such growth responses, although less pronounced, are observed in the presence of an excess of riboflavin, provided sufficient lyxoflavin is fed. Such growth responses cannot be explained on the basis of present knowledge. They may result from an antibiotic-like action of lyxoflavin, or be akin to the unexplained growth-stimulating effects observed in microorganisms when a variety of toxic compounds are present in subinhibitory concentrations. The large amounts of lyxoflavin required to obtain such responses, coupled with the evidence against the natural

occurrence of this compound, make it highly improbable that the compound functions as a true vitamin.

Summary. 1. L-lyxoflavin, D-galactoflavin and isoriboflavin markedly increase the growth response of *Lactobacillus casei* to suboptimal amounts of riboflavin, but do not promote growth in the absence of this vitamin. Appropriate differential assays indicated that lyxoflavin was not deposited in cells of *L. casei* under conditions where it stimulated growth, and actually decreased the concentration of riboflavin deposited in such cells. Thus it does not serve as a partial substitute for riboflavin in *L. casei,* but appears to enhance the efficiency with which limited supplies of riboflavin are utilized. When riboflavin is present in excess, lyxoflavin does not increase the cell yield or the growth-rate. At higher concentrations, lyxoflavin and galactoflavin inhibit growth by acting as competitive antagonists of riboflavin. Dichlororiboflavin and isoriboflavin are ineffective as inhibitors of this organism. 2. L-lyxoflavin promotes maximum growth of *Lactobacillus lactis* in the absence of riboflavin, and such growth continues indefinitely upon subculture. Differential assay with *L. casei* and *L. lactis* showed that cells of the latter organism grown with lyxoflavin contain no riboflavin, but do contain lyxoflavin; *i.e.,* in this organism lyxoflavin can fill the essential metabolic roles normally played by riboflavin. Similar assays of tissues from rats grown on natural rations indicate that lyxoflavin does not occur naturally in significant quantities. 3. In chicks, lyxoflavin stimulated growth when fed at levels 2.5 to 10 times the amount of riboflavin in the diet. At ratios of lyxoflavin to riboflavin higher than 40 to 1, lyxoflavin inhibited growth. Amounts of lyxoflavin that inhibited growth on diets of low riboflavin content were non-toxic when the amount of riboflavin was increased. 4. Although the growth-responses observed in chicks when L-lyxoflavin is added to diets low in riboflavin may result in part from a sparing action similar to that found with *L. casei,* such a sparing action does not explain the growth effects of the analog observed in rations of high riboflavin content. Relatively large amounts of lyxoflavin are required for such growth stimulation, and since available evidence indicates that the compound does not occur naturally, it cannot be considered a vitamin.

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Effect of Motor Cortex Ablations on Reflex Myoclonus in the Monkey. (20187)

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We have previously reported(1) that ablation of the motor cortex in the cat produces a marked depression of the myoclonic responses of the affected extremities, as elicited by afferent stimulation after administering subconvulsive doses of metrazol. These studies have been extended to the macaque for two purposes: 1. To ascertain if the motor cortex plays a similar role in the myoclonic mechanism in a primate. 2. To be able to more sharply delimit the cortical ablations than was possible in the cat and to avoid implication of non-motor frontal ("pre-frontal") cortex.

Methods. Experiments were performed on 5 mature rhesus monkeys (*Macaca mulatta*). For studies of the reflex myoclonic responses, the animals were anesthetized with pentobarbital (nembutal) 50 mg/kg intraperitoneally. In one experiment, dial 45 mg/kg was used. Electrical activity of the brain was recorded either through phonograph needles embedded in the skull or through wick electrodes placed on the cortex. Electromyograms were recorded from bipolar needle electrodes placed symmetrically in the limb pairs. All records were made by a Grass 8-channel, ink-writing oscillograph. Sub-convulsant doses of metrazol, 5 to 10%, were administered by slow intravenous injection during continuous recording. The physiologic stimulus was a click produced in an earphone by the square-wave output of a Grass stimulator. The usual frequency of repetition of the click was 0.5/sec. Cortical ablations were performed with suction and cautery as follows: 1. Acute ablation of pre-central gyrus of one side—one monkey. 2. Acute ablation of one arm area—one monkey. 3. Chronic ablation of one arm area—2 monkeys. 4. Chronic ablation of one leg area—one monkey. Before performing the limited ablations, the excitable cortex of the pre-central gyrus was identified by stimulation, dial, 40 mg/kg, being used for anesthesia in all except the first preparation. In the chronic preparations, the ablations were performed aseptically and the animals were kept for 6 to 7 weeks, at which time they had attained an essentially static level of recovery of function, and showed only slight awkwardness in movement in the distal portion of the affected extremity. At the end of this period, the animals were prepared for study of the reflex myoclonic responses, as described above.

Results. The results of all experiments were essentially similar. Ablation of the motor cortex, in whole or in part, resulted in complete or partial failure of the affected extremities to participate in the myoclonic response to acoustic stimulation. In the case of the ablation of the whole pre-central gyrus, the affected upper and lower extremities both showed depression of the myoclonic response. With the ablations limited to arm or leg area,

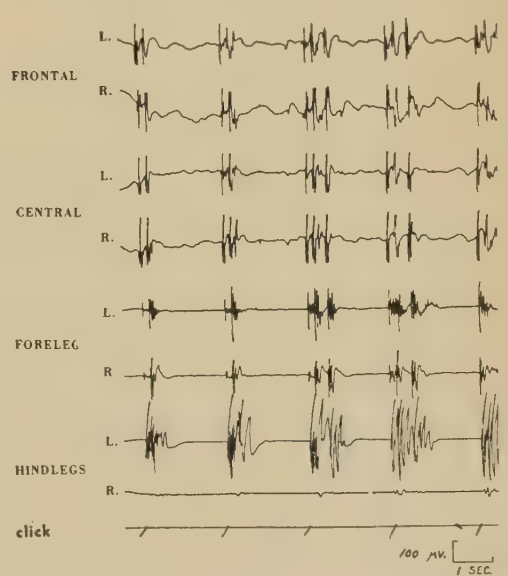

FIG. 1. Ablation of left hind-leg area performed 6 wk previously (lesion shown in Fig. 2). Note the almost complete absence of response of the right hind-leg.

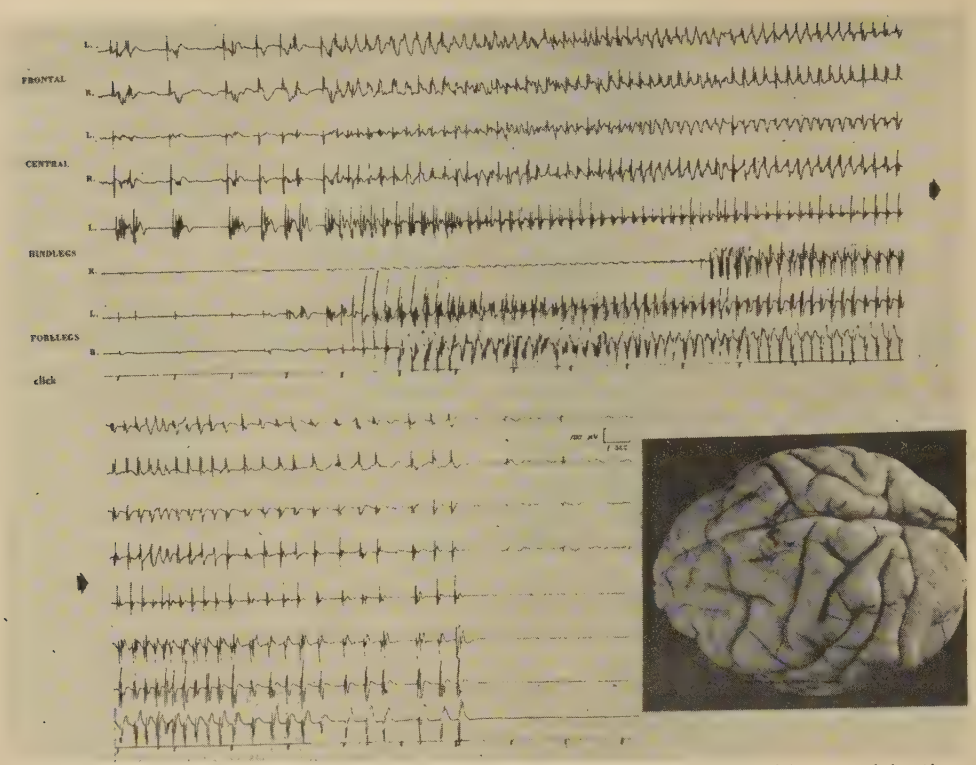

FIG. 2. Same preparation as in Fig. 1. Generalized seizure, with right hind-leg participating, but only after a delay of more than 14 sec.

the single extremity showed depressed response (Fig. 1).

Frequently, the depression was complete, no myoclonic responses of the affected extremity being recorded. On occasion, the depression was only partial, being manifested by a marked asymmetry of response between the two sides. The myoclonic responses of the paretic extremities, when they did appear, also showed a difference in pattern, being confined mostly to the proximal muscles of the limb; there was absence or marked diminution of the finger-flexor movements observed prominently in the normal extremity. In one chronic preparation, stimulation at the time of the acute experiment demonstrated that not all the primary cortical motor representation of the upper extremity had been ablated. Nevertheless, this animal showed marked depression of the myoclonic response, as was demonstrated in the animals with complete ablation.

With increasing dosage of metrazol, grand mal seizures occurred spontaneously or could be induced by repetitive acoustic stimulation. During such seizures, the affected extremities were at first quiescent, but after a variable period, became involved in the seizure activity, sometimes beginning with almost no buildup, almost as if a switch had been thrown (Fig. 2). During a seizure the movements of these extremities showed a difference in pattern similar to that observed during single myoclonic twitches, with diminished finger and toe-flexor activity and with the extensors of the limb more active than the flexors.

Discussion. The findings of depression or absence of the myoclonic response consequent upon limited ablation of motor cortex in the monkey are in accord with those previously reported in the cat with more extensive frontal ablation.

Although not studied in the monkey, it has been demonstrated in the cat that myoclonic responses may still be obtained after bilateral ablation of motor cortex, although there is a great increase in threshold, *i.e.*, considerably more metrazol is required(1). Unilateral

ablation of all cortex except the frontal pole does not influence the responses. Bilateral, but not unilateral, ablation of specific sensory cortex has been reported to abolish these responses(2,3), but evidence has been presented demonstrating that this too represents only a rise in metrazol threshold, involving only the specific sensory system whose cortical representation has been ablated(4). In the case of the motor cortex, with unilateral ablation there is a depression of the response to all forms of stimulus.

It is evident that the motor cortex contributes but is not essential for reflex myoclonus. This infers that in some way the motor cortex exerts a facilitatory action on the neuronal mechanisms involved in the response. Whether this action is exerted at a spinal or supraspinal level is not yet established. Preliminary observations indicate the probability that metrazol has little influence on the spinal mechanisms in the nembutalized cat(5). If this be true, it is evident that the facilitatory influence of the motor cortex is to be attributed to activity at a supraspinal level, through its extrapyramidal projections.

The actual site and mechanism of action of metrazol are unknown. It is of interest that the myoclonic twitch of metrazolized man or animal is primarily a bilateral flexor response. Ablation of the motor cortex markedly diminishes these responses, and the participation of the affected extremity in a generalized seizure is characterized by a relative sparing of extensor mechanisms. The neuronal basis of such specificity of metrazol action is under investigation at the present time.

Summary. 1. Sharply limited ablation of the arm or leg area of the precentral motor cortex in the macaque produces a complete or partial depression of the reflex myoclonic responses of the affected extremities as elicited after subconvulsant metrazol dosage. This occurs in chronic, as well as acute preparations. 2. When responses are present, there is a predominance of extensor muscle activity, with marked diminution of finger- or toe-flexor responses. A similar pattern is observed when generalized seizures are elicited by repetitive acoustic stimulation.

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Estimation of Vi Antibody Employing Erythrocytes Treated with Purified Vi Antigen.* (20188)

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The occurrence of Vi antibody in the sera of individuals harboring typhoid bacilli was first observed by Felix(1,2). Reports from many laboratories have confirmed and generally established this relationship(3-5). Consequently, the presence of Vi agglutinins is now commonly considered to be correlated with the harboring of typhoid bacilli and may serve as a satisfactory means of detecting the carrier state. As a result of its relative simplicity and because it is a more practicable method than the bacteriological examination of stool specimens, the test for Vi antibody has been employed extensively as a screening procedure for the detection of typhoid carriers in large populations. The method enjoying the widest

* Presented at 79th Meeting of the American Public Health Assn., San Francisco, Calif., Nov. 1951.

acceptance employs the Vi I strain of *S. typhosa* (Bhatnagar)(6). This culture is considered a pure Vi variant of *S. typhosa* free of H antigen and containing only a very small amount of O antigen, insufficient to interfere with its Vi agglutinating activity. In practice, however, it has been reported that the Vi I strain may yield an appreciable percentage of false positives(7) and most reports concerning its use emphasize the need for great care in its maintenance to obtain reliable results. While the value of the Vi agglutination test for the detection of typhoid carriers no longer is seriously questioned, it has been modified frequently by many workers in the field and the technic of its performance has not been fully standardized, although more than a decade has elapsed since its inception. The large number of publications concerning the technic, together with the fact that properties of Vi agglutination suspensions and the details of the test procedure differ widely, are all indicative of decided variations in the specificity of the test in the hands of various workers(5) and suggest that the procedure employed for determination of Vi antibody may be susceptible to improvement. Vi antigen was recently isolated by Webster, Landy, and Freeman(8) from V form *Escherichia coli* 5396/38, a non-typhoid enteric species rich in this antigen, by an ethanol-salt fractionation procedure, and it was shown to be a polymeric acid. Landy and Webster(9) in a study of its immunological properties, presented evidence indicating that the Vi antigen in the purified state had retained its distinguishing serological and immunogenic characteristics. Among other immunological attributes it was demonstrated that the purified Vi antigen could be adsorbed on human type O erythrocytes which then became specifically agglutinable by Vi antisera. This observation suggested that the isolated Vi antigen, possessing high potency and stability, might offer certain advantages in coping with the practical problem of detecting or measuring Vi antibody. It is the objective of this report to present a procedure for the preparation of a Vi agglutination reagent of high serological activity by the sensitization of human type O erythrocytes with purified Vi antigen and to indicate the results obtained with its use in the estimation of Vi antibody.

Materials and methods. Vi bacterial agglutination test. The Vi antigen suspension made from *S. typhosa* Vi I (Bhatnagar) and Vi antiserum† distributed by the Standards Laboratory of the Central Public Health Laboratory, London, were employed in accordance with the laboratory's procedure for conducting the Vi agglutination test. Agglutination tests were also performed with viable suspensions of *S. typhosa* Vi I freshly prepared for each test and checked with a reference (*ballerup*) Vi serum. These tests yielded essentially similar results. *Preparation of Vi antigen.* The purified Vi antigen employed in this investigation was prepared from acetone killed and dried V form *E. coli* 5396/38 by the method described by Webster *et al.*(8). Solutions of the antigen containing 10 μg per ml in 0.85% NaCl were stored at 4°C with 1:10,000 merthiolate as preservative. *Collection and preservation of erythrocytes.* Human type O erythrocytes were collected in equal volumes of modified Alsever's solution.‡ Erythrocytes were washed 3 times in 10 volumes of physiological saline, centrifuged each time in conical tubes at 1500 RPM for 5 minutes, and the supernatants discarded. The packed erythrocytes were resuspended in 10 volumes of saline and centrifuged at 1500 RPM for 10 minutes. The supernatant was discarded and the packed cells made up to a 10% suspension. *Sensitization of erythrocytes with purified Vi antigen.*§ Equal volumes of 10% erythrocytes and a solution

† The Vi reference serum (horse) distributed by Central Enteric Reference Laboratory for use in this test exhibits a Vi hemagglutination titer of approximately 1:10000. The serum dilution of 1:1400 is considered the "endpoint" for purposes of reading the results of the Vi bacterial agglutination test.

‡ Blood drawn by venipuncture was added to a flask containing an equal volume of sterile modified Alsever's Solution (dextrose 2.05%, NaCl 0.42%, trisodium citrate 0.055%). Contents were mixed and dispensed aseptically as 3 ml aliquots into sterile vaccine bottles fitted with rubber sleevecaps and stored at 4-8°C. Under these conditions of collection and storage, erythrocytes suitable for hemagglutination tests may be retained for at least one month.

of Vi antigen (10 μg per ml in saline)‖ were mixed and incubated in a water bath at 37°C for 2 hours with shaking every 30 minutes during incubation. Excess or unabsorbed Vi antigen was inhibitory and was removed by washing the suspension 2 times with 10 volumes of saline, centrifuging in conical tubes at 2000 RPM for 5 minutes and discarding supernatants. The packed cells following the second washing were made up to 10 times the original volume (cells plus antigen solution) and centrifuged at 2000 RPM for 10 minutes. The supernatant was discarded and the packed cells made up to a 1% suspension. When refrigerated at 4°C sensitized erythrocytes retain their specific agglutinative properties and are satisfactory for use in this test for 2 days.

Vi hemagglutination test. The test was set up to include reference Vi serum, known negative serum and serum and saline controls. Sensitized erythrocyte suspension (0.1 ml) was added to 0.2 ml volume of serial 2-fold dilutions of the test sera in 10 x 75 mm agglutination tubes. The tubes were shaken and incubated in the water bath at 37°C for 2 hours. Agglutination was determined by examining the pattern of settling of the erythrocytes on the bottom of the tube after the manner of Salk's technic for the titration of influenza hemagglutination. In the tubes containing a high concentration of immune serum a positive (complete) agglutination pattern appeared early and was most marked. This was in the form of a compact mass with ragged edges. Tubes containing lower concentrations of immune serum but sufficient to yield complete or almost complete agglutination exhibited a dispersed pattern of cells entirely covering the bottom of the tube. Tubes containing a still lower concentration of immune serum, and normal or negative sera, revealed a "doughnut" shaped pattern or a compact "button" of cells. The titer was expressed as the final dilution of serum in the last tube which showed complete or almost complete agglutination. This tube was the one immediately preceding the first tube showing a "doughnut" pattern of cells.

Results. It was considered desirable to compare the results of Vi antibody titrations by the procedure outlined above with the results obtained by other methods. Accordingly, hemagglutination titrations were made on 11 rabbit Vi antisera on which Vi bacterial agglutination titers and antibody N precipitable with the purified Vi antigen had been determined previously. The results of these tests are summarized in Table I.

The ratios of the hemagglutination to the agglutination titers and antibody N values for these sera are tabulated to indicate the extent to which these measurements of Vi antibody content are related. It will be noted that the ratios of the hemagglutination titers to the bacterial agglutination titers varied from 4.0 to 10.5, while the ratios of the antibody N values to hemagglutination titers ranged from 1.9 to 6.6. These respective 2.6 and 3.5 fold variations obtained for this group of Vi antisera are moderate and are only slightly in excess of the experimental error common to tube agglutination tests. These results indicate that the three determinations, one of which is known to possess analytical accuracy, are correlated.

The application of the Vi hemagglutination technic to the measurement of antibody reactive with the purfied Vi antigen was studied with a variety of human sera which may be divided into four groups: normal, from individuals vaccinated with heat-killed typhoid vaccine, from individuals immunized with the purified Vi antigen and from typhoid carriers.

§ It was established that Vi antigen could also be adsorbed on erythrocytes of the mouse, guinea pig, rabbit, dog, sheep and horse. Of these species erythrocytes from sheep and man required the smallest quantity of antigen for sensitization. Human erythrocytes were employed exclusively in this study since, with their use, it was not necessary to absorb test sera for removal of normal hemagglutinins. For most laboratories they offer the added advantage of being more readily available than sheep erythrocytes.

‖ Serological activity of purified Vi antigen, *i.e.*, antigen titration, has been recorded elsewhere(9) and need not be repeated here. The quantity of Vi antigen suggested for sensitization of erythrocytes represents an amount considerably in excess of the "antigen titer". However, provided the antigen treated erythrocytes are washed, this excess is not inhibitory and provides a wide margin of safety in terms of sufficient antigen for maximum reactivity.

TABLE I. Correlation of Bacterial Agglutination, Quantitative Precipitin and Hemagglutination Determinations on Rabbit Vi Antisera.*

Hemagglutination titer	Agglutination titer	Hemagglutination titer / Agglutination titer	Antibody N/ml, μg	$\frac{\gamma \text{ antibody N}}{\text{Hemagglutination titer}} \times 100$
3210	680	4.7	215	6.6
6720	720	9.3	296	4.4
3210	400	8.0	115	3.5
960	140	6.8	28	2.9
840	80	10.5	16	1.9
940	150	6.3	33	3.5
600	150	4.0	27	4.5
3210	460	6.9	128	3.9
840	170	4.9	32	3.8
840	210	4.0	48	5.7
1680	170	9.8	46	2.7

* Rabbits were immunized with 1.5 mg of acetone killed and dried V form *S. typhosa*.

TABLE II. Results of Vi Hemagglutination Tests on Human Sera.

Sera	Normal: No history of typhoid or of typhoid immunization	Vaccinated: Received 3 inj. of heat killed typhoid vaccine	Vi immunized: Received single inj. of 40 μg purified Vi antigen	Typhoid carrier: Confirmed by isolation of *S. typhosa*
Total	252	251	220	20
No. positive Vi titer	4	18	213	19
% positive Vi titer	1.6	7.1	96.8	95

The results of these tests are given in Table II.

The 1.6% positive Vi reactors detected in normal individuals was in good agreement with the findings of 3.0% by Klein(5) and 2.9% by MacKenzie and Taylor(10). However, the incidence of Vi positives found in individuals immunized with heat killed typhoid vaccine (7.1%) was somewhat higher than the figure of 4.5% obtained by MacKenzie and Taylor. It generally has been assumed that the heat killed typhoid vaccine fails to elicit the production of Vi antibody. This probably is true if one considers the consistent failure to demonstrate Vi antibody by the use of the conventional bacterial agglutination tests. However, these results obtained with a more sensitive and specific test suggest that small, but measurable, amounts of Vi antibody occasionally are encountered in individuals immunized with heat killed-phenol preserved typhoid vaccine. This point requires further study.

Almost all individuals who received a single subcutaneous injection of 40 μg of purified Vi antigen and were bled 2 weeks later were found to possess antibody reactive with this antigen when their sera were tested by the hemagglutination technic(11). Eleven of the subjects possessed Vi antibody prior to immunization. However, all exhibited a significant rise in Vi titer following immunization.

Vi antibody was detected in the sera of 19 of 20 typhoid carriers tested by the hemagglutination procedure. The detailed breakdown of the figures on these sera and the comparison of titers with the Vi bacterial agglutination test in shown in Table III. Inspection of these data indicates that except for serums 19 and P-51, the bacterial agglutination and the hemagglutination tests were comparable in the detection of Vi antibody in the sera of typhoid carriers. However, the titers obtained with the hemagglutination test ranged up to 30 fold higher than the titers observed in the bacterial agglutination test. No explanation can be given at this time for these variations in Vi titers by the hemagglutination procedure. In contrast to this variation, it was observed that sera from individuals injected with purfied Vi antigen and tested by the two methods yielded values which were considerably more uniform. Table

TABLE III. Vi Antibody Determinations on Sera from Chronic Typhoid Carriers.

Bacteriologically proved duration of carrier state	Type*	Vi antibody titer (reciprocal) determined by: Bacterial agglutination test	Vi antibody titer (reciprocal) determined by: Hemagglutination test
30 mo	FT	40	30
29	UT	80	960
22	"	5	15
22	"	60	1920
24	"	80	240
23	"	5	Neg
23	"	40	120
22	"	80	240
21	"	30	240
22	"	160	2560
22	UC	20	60
23	UT	50	240
n.k.*	FT	Neg	7.5
n.k.	"	80	480
n.k.	"	20	120
n.k.	"	10	60
n.k.	"	20	60
10 yr	"	10	60
n.k.	"	Neg	Neg
4 mo	"	40	120

* Type FT, fecal typhoid; UT, urinary typhoid; UC, urinary paratyphoid C; n.k., not known.

Sera were kindly supplied by the late Surgeon Commander W. S. Miller, R. N., U. S. Naval Medical Research Unit No. 3, Cairo, Egypt; Dr. Daniel Widelock, Department of Health, City of New York and Dr. C. Gentzkow, Director, Bureau of Laboratories, Philadelphia, Pennsylvania.

TABLE IV. Vi Antibody Titers of Human Sera* Tested by the Hemagglutination and the "Standard" Bacterial Agglutination Procedures.

Vi titer by hemagglutination test	No. of sera tested	Reciprocal of "standard" Vi agglutination titer: 5	10	15	20	30	40	60	80
1:30	8	4	3	1					
1:60	6		1	4	1				
1:120	7				3	3	1		
1:240	6						1	3	2

* Individuals inj. subcut. with 40 μg of purified Vi antigen and bled 3 wk later.

IV gives the results of these comparative tests. Four groups of sera which had exhibited Vi titers of 1:30 to 1:240 by the hemagglutination procedure were tested by the conventional bacterial agglutination test. It will be noted that in every instance lower Vi titers were obtained and that these ranged from 1/3 to 1/6 of those recorded for the more sensitive hemagglutination test. However, the tests generally parallel one another with the hemagglutination test providing the more sensitive measure of Vi antibody.

The data presented are indicative, in a preliminary way, of what may be expected of the Vi hemagglutination technic for the estimation of Vi antibody. The most serious limitations of the Vi bacterial agglutination test have been the lack of a stable Vi agglutination suspension and the pronounced variability in the sensitivity of suspensions from lot to lot. British investigators, notably Felix and his associates at the Central Enteric Reference Laboratory and Bureau in London, have sought to standardize the Vi agglutination test by the use of a standard or reference Vi serum and issuance of a "standard" TVi suspension. In so doing, they have materially improved the performance of the test. However, little or no progress has been made in the development of a stable Vi antigen suspension and the product most widely used today (British) bears a 60-day expiration date.

On the other hand, the purified Vi antigen prepared from *E. coli* is a uniform product, is stable indefinitely and is reactive with Vi antibody only. The possibility of "non specific" reactions has been, as far as can be determined, eliminated. The potency of the product is so great that a single lot of 2 g, such as is presently prepared in these laboratories, would suffice for the sensitization of erythrocytes for 20 million tubes in the Vi hemagglutination test. Human type "O" erythrocytes are readily obtainable, may be preserved in Alsever's solution for periods of at least 30 days and give uniformly consistent results independent of their donor. The sensitization of erythrocytes with Vi antigen is a rapid and convenient procedure and requires only simple serological apparatus and technic.

Summary. 1. The preparation of a sensitive and specific Vi agglutination reagent by sensitization of human type O erythrocytes with Vi antigen isolated from *E. coli* has been described. 2. Vi antibody content of rabbit and human sera was determined by this method and the conventional bacterial agglutination test. The hemagglutination test provided a more sensitive measure of Vi antibody by a factor of 3 to 6 fold. Greater variability in Vi titers was encountered in testing the sera

of typhoid carriers; however, both methods were in good agreement in the detection of Vi antibody in these sera. The advantages inherent in the Vi hemagglutination procedure are discussed.

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Effects of p-Aminosalicylic Acid on Thyroid and Adrenal.* (20189)

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Recent clinical observations have indicated that goiter and, rarely, transient myxedema may occur in patients treated with p-aminosalicylate (PAS) over prolonged periods(1-3). Bavin(4) and Kjerulf-Jensen and Wolffbrandt (5) have shown that rats fed PAS developed cellular hyperplasia of the thyroid gland, and Rosenberg(6), in a study of a large group of peroxidase inhibitors, has reported diminished I^{131} uptake in rat thyroid glands after a single parenteral dose of PAS. Similarly, Hanngren (7) has reported a reduction in thyroid I^{131} uptake in 3 patients given a single intravenous injection of this compound.

Investigations over the past few years have suggested that salicylic acid and similar compounds exert a stimulatory effect on the pituitary-adrenal axis. Cronheim *et al.*(8) and Van Cauwenberge *et al.*(9) have shown that the administration of salicylic acid, aspirin and sodium salicylate to rats results in a marked eosinopenia and reduction of adrenal ascorbic acid and cholesterol concentrations. Whereas Cronheim did not observe these changes in the case of PAS, Van Cauwenberge reported a fall in circulating eosinophiles and ascorbic acid and cholesterol concentrations following a single parenteral dose of this compound. Bavin(4) on the other hand, found no eosinopenic response in mice following PAS administration. Because of these conflicting results and the widespread use of prolonged PAS therapy in tuberculosis, it was felt that further evaluation of the effects of prolonged administration of PAS on rat thyroid and adrenal functions was indicated.

Experimental. Male Sprague-Dawley rats weighing approximately 180 g were used. They were kept at a constant room temperature of 80°F, and were fed standard Purina chow diet, modified as indicated. All rats were weighed at the beginning and at the end of the experiment. The animals were divided into 4 groups of 10 rats each. One group of rats was fed 1% powdered PAS in the diet for 2 weeks and control and comparison groups were given a) stock diet alone, b) stock diet containing 0.2% powdered propylthiouracil and c) stock diet plus daily subcutaneous injections of 1 mg of ACTH gel.† At the end of the 2-week period, a tracer dose of I^{131} (6 μc) was injected intraperitoneally into all animals and at the same time food was removed from the cages. Four hours after in-

* Aided by a grant from the U. S. Public Health Service and Research Fund 171.

† Kindly supplied by Dr. Grosvenor Bissell of the Armour Laboratories, Chicago, Ill.

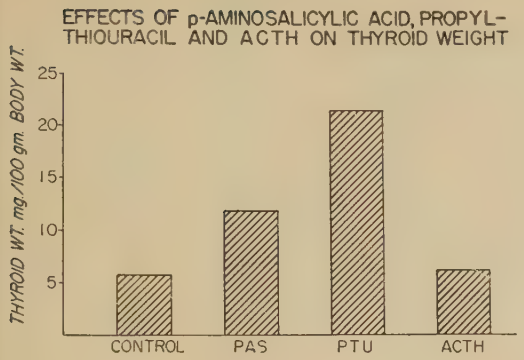

FIG. 1.

jection the animals were anesthetized with ether and blood removed from the aorta. The thyroid glands were weighed and the radioiodine content of the glands was determined by means of a counting system employing a gamma well counter. In addition, the following determinations were made: adrenal weight, adrenal ascorbic acid(10) and cholesterol(11) concentrations; thymic weight, liver glycogen concentration(12) and circulating eosinophiles(13). An experiment similar to the above was carried out on two groups of hypophysectomized male rats.‡ One group received 1% PAS in the diet and the other group served as a control.

Results. All animals studied showed a comparable weight gain and appeared healthy at the end of the 2-week period. PAS given as 1% of the diet, was found to exert a marked goitrogenic effect (Fig. 1), though not as great as propylthiouracil, given as 0.2% of the diet. The thyroid glands of the PAS group averaged 12 ± 0.42 mg/100 g of body weight (mean ± standard error), whereas those of the controls weighed 6 ± 0.27 mg/100 g body weight. This difference is very highly significant ($P < .001$) and constitutes a 2-fold increase in size of the thyroid following PAS therapy. The thyroid glands of the rats given propylthiouracil weighed 3 times as much as those of the controls, averaging 21 ± 0.78 mg/100 g body weight. The weights of the thyroids of the ACTH-treated animals did not vary significantly from those of the controls. The radioiodine concentration in the thyroid glands of PAS group was reduced to 20% of that of the controls, and the total I^{131} content of these hypertrophic glands was 40% of the controls (Fig. 2). No significant difference in thyroid I^{131} concentration was observed between the PAS and propylthiouracil-treated animals, although the larger thyroids of the thiouracil group accumulated more total radioiodine. Again, no significant change was noted in the thyroids of the ACTH group.

Whereas the thyroids of the PAS-fed hypophysectomized animals contained only 17% as much radioiodine as the hypophysectomized controls, no thyroid enlargement was noted, the glands weighing approximately the same in both groups (Fig. 3).

No differences in adrenal weight, adrenal ascorbic acid and cholesterol concentrations, circulating eosinophiles and thymic weight were observed in any of the 4 groups studied (Table I). However, elevation of liver glycogen concentrations in PAS-treated animals was noted. The changes are shown in Fig. 4. Whereas the livers of the controls contained 42 ± 2.25 mg glycogen/g of liver, those of the PAS animals contained 58 ± 3.35 mg/g ($P < .001$). This observation was also made in hypophysectomized animals, where liver

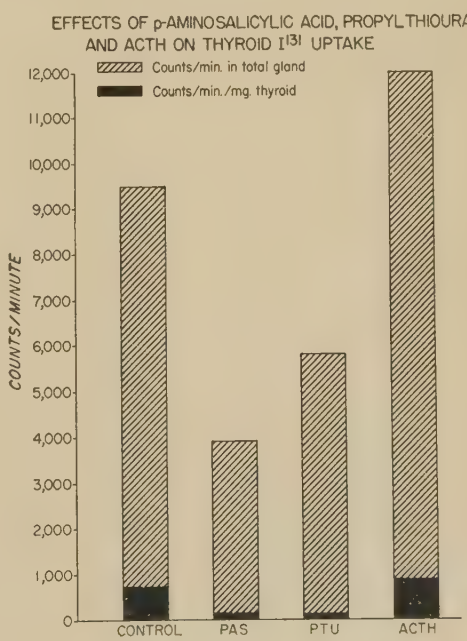

FIG. 2.

‡ Obtained from the Endocrine Laboratories, Madison, Wisc.

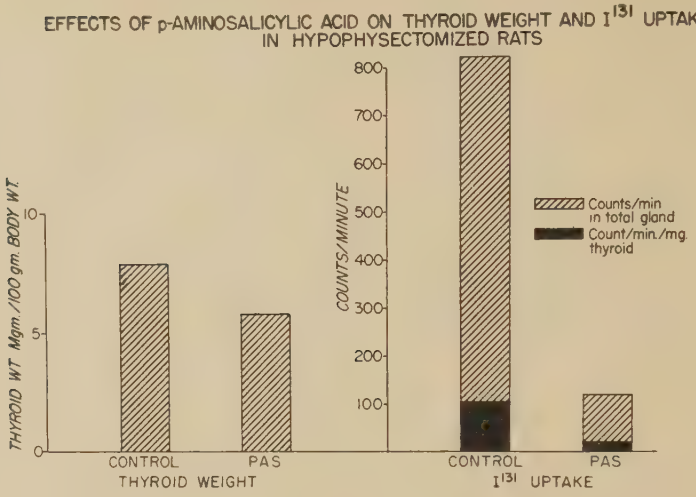

FIG. 3.

glycogen concentrations in the controls were 1.2 ± 0.69 mg/g of liver and those in PAS animals were 19 ± 6.52 mg/g (Fig. 4). This difference is also significant (P 0.02).

Discussion. These studies showed that the administration of PAS results in goiter and inhibition of I^{131} uptake by the thyroid gland in rats, presumably by a mechanism similar to that postulated in the case of propylthiouracil. Inhibition of iodine concentration by the gland results in a decreased formation and secretion of thyroxine with consequent increased secretion of thyrotropic hormone by the anterior pituitary. This in turn, results in thyroid stimulation and enlargement. This explanation is supported by the findings in hypophysectomized animals, where thyromegaly was not observed as a result of PAS ingestion, although diminution in I^{131} was. Further substantiation of this thesis comes from observations by Kjerulf-Jensen and Wolffbrandt that the goitrogenic effect of PAS could be prevented by the administration of thyroid extract. 1% PAS in the diet produced less thyromegaly than did a diet of 0.2% propylthiouracil, indicating that PAS is not as potent a goitrogenic agent as propylthiouracil though the type of pharmacological effect on the thyroid gland is probably the same in both cases. The diminished I^{131} uptake by the thyroid gland following ACTH and cortisone administration reported by Hill *et al.*(14), and Berson(15) was not observed in our experiments. However, it must be kept in mind that the animals in our series received the last injection of ACTH gel 24 hours be-

TABLE I. Effects of *p*-Aminosalicylic Acid, Propylthiouracil and ACTH on Adrenal Properties Function.

Group	Adrenal wt, mg/100 g body wt	Adrenal ascorbic acid, mg/100 mg gland	Adrenal cholesterol, mg/100 mg gland	Thymus wt, mg/100 g body wt	Eosinophil count, per mm^3
Control	7.71*	0.483	3.40	206	16.9
PAS	7.52	0.419	3.90	202	17.5
Propylthiouracil	7.43	0.490	3.50	159	14.2
ACTH	9.43	0.655		191	19.2
Hypophysectomized control	4.90	0.364		197	11.7
Hypophysectomized PAS	3.94	0.340		146	23.3

* All values are the mean of individual determinations on 10 animals.

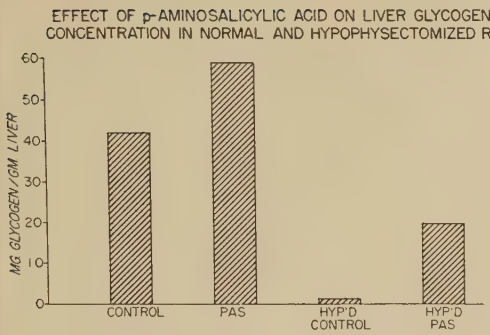

FIG. 4.

fore the radioiodine tracer dose was given.

Our findings substantiate the observation of Cronheim *et al.* that PAS has no effect on the pituitary-adrenal axis, at least when administered over a 2-week period. Although elevation of the liver glycogen concentration was observed in all PAS-treated animals, this is not necessarily an adrenal-mediated response and may be the result of a direct action of PAS in the liver; this is especially interesting in view of the report by Zahn(3) that many patients receiving PAS excrete a reducing substance in their urine. Further work on this phase of the problem is contemplated.

Summary. 1. Administration of PAS to rats over a 2-week period produced goiter associated with diminished I^{131} uptake by the thyroid gland. 2. No effect on the pituitary-adrenal axis was observed. 3. Elevation of liver glycogen concentration was noted in all PAS-treated animals.

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A Simple Direct Method for Absolute Basophil Leucocyte Count.* (20190)

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Little is known concerning the physiologic and pathologic significance of the basophil. Previous attempts to study these cells have been hindered by the scarcity of basophils in the bone marrow and circulating blood. Slight differences in absolute basophil counts are not easily detectable by indirect methods and at least 1000 leucocytes should be counted to insure a fair sampling of the basophils present. Such counts are also apt to have a large margin of error due to distribution of leucocytes in the stained blood smear. With a direct counting method, one in which the cells are tallied and differentiated in the same

* Presented at Am. Fed. for Clinical Research, 1952, New York City.

† John and Mary R. Markle Scholar in Medical Sciences.

counting chamber, these difficulties would not be encountered. Such a method is described in this paper.

The method employs the metachromatic staining principle described by Ehrlich(1), in which the metachromatic material takes a different color than might be expected of the stain. The granules in the cytoplasm of the basophil contain a metachromatic substance. Of the various metachromatic stains tested, toluidine blue appeared to be the most suitable and stained the basophil granules a bright pink to reddish-violet color. The nuclei of all leucocytes, including the basophil, stained bluish-violet differing only in intensity of staining as to the type of cell. The eosinophils can easily be distinguished by their larger size, the greenish-yellow appearance of their cytoplasm and granules, and by the rounded bi-lobed nuclei. The significant point is that only the basophils take a metachromatic stain.

Method. A diluent was needed which would destroy or hemolyze the erythrocytes and one which would not dissolve the water-soluble basophilic granules. Inorganic acids and bases, acetic acid, propylene glycol, and several short-chained alcohols were tested as diluents. Acetic acid and the inorganic acids were effective hemolyzing agents, but the basophilic granules were soluble in these reagents. Inorganic bases hemolyzed not only the erythrocytes, but destroyed the leucocytes as well. Propylene glycol was a good hemolyzing agent, but it failed to fix the basophilic granules(2). The short-chained alcohols in concentrations of 15-30% were effective in fixing the granules, but they were ineffective as hemolyzing agents. A solution of saponin in 20% ethanol was found to best meet the requirements of the diluent needed for the proper staining of these cells. Table I gives the composition of the fluid used in this study. Fresh unclotted blood obtained by finger puncture or citrated, or oxalated, venous blood is diluted with the above counting fluid in a proportion of one to 10 in the standard leucocyte pipette. The pipette is shaken either mechanically or manually for 3 minutes. The 4 chambers of 2 Fuchs-Rosenthal (0.2 mm deep) hemocytometers are filled by capillary action in the usual manner. The counting chambers are allowed to stand a few minutes so that the leucocytes may settle. They should be placed into a large covered Petri dish into which a dampened piece of blotting paper has been placed to insure against excessive drying, and while one hemocytometer is being counted, the other is left within the moistened atmosphere. The high magnification (4 mm objective) is used and all 4 chambers are counted. Not enough contrast between the metachromatic staining granules and the nuclei of the other cells is obtained to permit differentiation of the basophil under a low magnification (16 mm objective). The number of basophils counted in all 4 chambers is totaled, averaged, and multiplied by the dilution factor, 3.13, which gives the absolute count in terms of basophils per cubic millimeter. A total leucocyte count per cu mm may be done simultaneously by counting 2 of the 16 larger squares, averaging, and multiplying by 50. It has been found best to fill the chambers immediately after shaking for if the pipette stands longer than 30 minutes, a precipitate may appear which forms around the leucocytes making accurate counting impossible.

TABLE I.
Composition of Staining and Diluting Fluid.

	ml
0.05% toluidine blue in 0.85% saline	40
95.0% ethyl alcohol	11
Saturated sol. of saponin in 50% ethyl alcohol	1

Mix well and filter before use. This solution will keep at least 4 months at room temp. Occasional filtering may be necessary to keep the solution free from sediment.

Results. Satisfied that this method was reproducible, correlation was made between the direct (absolute) basophil counts and indirect basophil counts (calculated from the total leucocyte count, and from the percentage of basophils on the peripheral blood smear after counting 4000 leucocytes). The total white count was taken from the same chamber in which the basophil count was determined. This procedure was carried out on 31 individuals; 11 normal males, 9 normal females, and 11 individuals with miscellaneous diseases. Fig. 1 demonstrates this correlation.

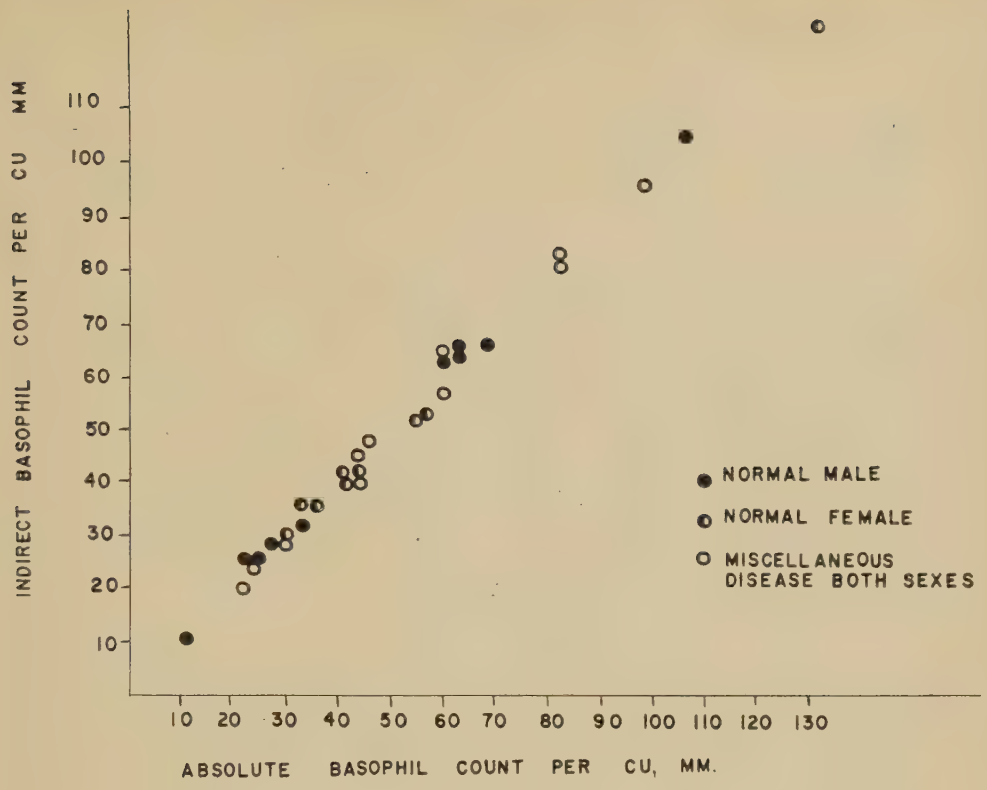

FIG. 1.

There appears to be an excellent agreement.

Normal values were determined on each of 36 young males and 33 females, all apparently healthy individuals who were either house officers, medical students, nurses, or technical assistants. In the male group, ranging in age from 20 to 38 years, the mean absolute basophil count was 46.7 ± 20.1 (S.D.) per cubic millimeter. The counts ranged from 11 to 107. In the female group, ranging in age from 18 years to 34 years, the mean absolute basophil count was 40.6 ± 18.9 (S.D.) per cubic millimeter. The total count ranged from 8 to 88 cells per cubic millimeter. There is no statistical difference between the means in these 2 groups. These data are summarized in Table II.

Discussion. This method now affords a means of studying changes in the absolute basophil counts in a variety of clinical disorders. Heretofore, the time-consuming necessity of counting at least 1000-2000 white cells has inhibited much needed investigation of the function and fluctuation in the basophil leucocyte. Changes in total basophils can be correlated with other physiological studies in the same manner as changes in the absolute eosinophils have been investigated. Studies of this nature are now in progress in this laboratory.

TABLE II. Normal Values for Basophils/mm³.

Sex	No.	Age	Mean	S.D.	Range
♂	36	20-38	46.7	±20.1	11-107
♀	33	18-34	40.6	±18.9	8-88

No significant sex difference t = .26

Summary. A method is presented which permits the differentiation and enumeration of basophils from the counting chamber. Basophil counts were performed on 69 normal individuals and the range, mean, and standard deviation are reported by sex.

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Renal Clearance of Pantothenic Acid in Man: Inhibition by Probenecid ('Benemid').* (20191)

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Probenecid has been shown to inhibit reversibly the renal tubular secretion of a number of organic acids, such as penicillin (1,2) phenolsulfonphthalein(3), and para-aminohippuric acid (PAH)(3,4). The plasma levels of para-aminosalicylic acid(5) and of the sulfonamides(6) also may be elevated by the coadministration of probenecid, but to a lesser degree than is the case with penicillin. The actual increase is small with PASA and sulfonamides and the mechanism by which this is accomplished has not been clearly demonstrated, but inhibiton of renal tubular secretion may be one of the mechanisms involved.

One of us (L.D.W.)(7) had previously studied the renal clearance of pantothenate in dogs, showing that with the 100-fold range of dosage employed, the clearance of pantothenate was not significantly greater than exogenous creatinine clearance. On the other hand, Roholt and Schmidt(8) in Sweden, who investigated pantothenate clearances in man over a wide range of plasma concentrations, reported pantothenate clearance to be significantly greater than inulin clearance at high plasma concentrations. At still higher plasma levels, a self-depression of pantothenate clearance or secretory Tm phenomenon was observed. This group has reported an inhibition of pantothenic acid clearance with carinamide.† Inasmuch as probenecid effects, in terms of a number of reference substances, have been shown to parallel those reported for carinamide, we were interested to see to what degree probenecid would inhibit the renal tubular secretion of pantothenic acid.

Methods. The pantothenic acid content of plasma and urine was determined by the microbiological method of Skeggs and Wright (9) and expressed in terms of calcium pantothenate. As a measure of glomerular filtration rate, urine and plasma specimens were analyzed by a modification of the Folin-Wu method(10) for endogenous materials that yield color with alkaline picrate. Endogenous chromogen clearance values are slightly lower than true creatinine clearances but Brod and Sirota indicate that chromogen clearance is a useful clinical test of filtration rate in man(11). The "creatinine" clearances referred to in this study are actually chromogen clearances. Since little, if any, chromogen is excreted by tubular secretion when plasma levels are in the normal range, the chromogen clearance may be regarded as an approximate measure of glomerular filtration rate even when probenecid is given concurrently. The plasma and urine PAH determinations were done by the method of Smith and associates(12). Our observations are presented in 2 sections, thereby conforming to the pattern of the investigation. The plan of study will be presented along with the results for each portion of the work.

Probenecid effect upon plasma pantothenate levels. In order to determine the loading dose required to attain plasma levels of pantothenate that would be associated with significant renal tubular secretion, sodium pantothenate was injected rapidly (2 to 3 minutes) by the intravenous route. Of the individuals presented in Fig. 1, L.B. received 500 mg and M.J. received 1000 mg of sodium pantothenate on each of two occasions: alone, and one hour after an intravenous injection of 2.0 g of probenecid. Comparison of the plasma values of pantothenate after the administration of pantothenate alone with those after pantothenate and probenecid at one, 2, and 3 hours after injection showed clearly that probenecid retarded the rate at which the

* Probenecid is the generic name for p-(di-n-propylsulfamyl)-benzoic acid, which is marketed under the trademark 'Benemid' by Sharp and Dohme, Inc.

† Carinamide is 4′-carboxyphenylmethanesulfonanilide, formerly marketed under trademark 'Staticin' by Sharp and Dohme, Inc., Philadelphia, Pa.

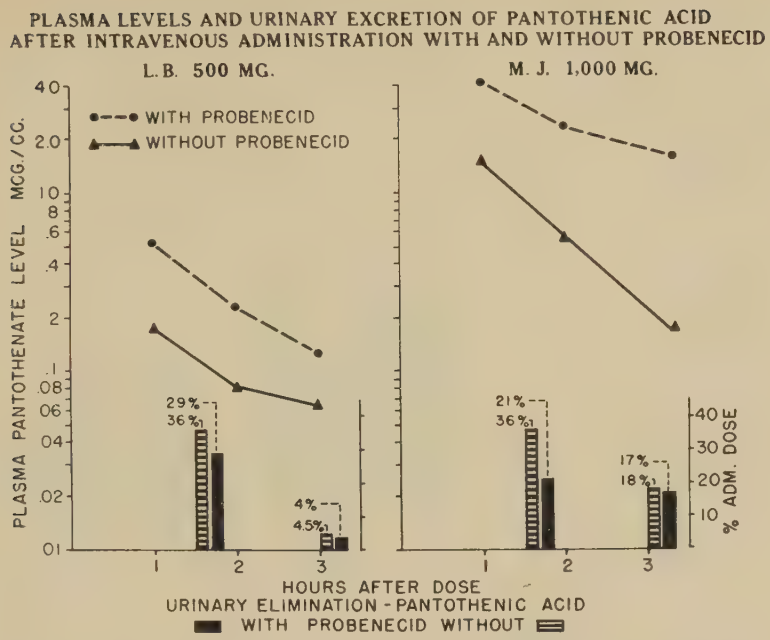

FIG. 1. Probenecid, 2.0 g intravenously, produced in two patients a 2- to 10-fold increase of pantothenate plasma concentrations over a 3-hr period after the intravenous administration of 500 and 1000 mg of sodium pantothenate respectively. Urinary pantothenate recoveries measured simultaneously showed a slight depression under the influence of probenecid.

plasma pantothenate levels declined. The urinary excretion of pantothenic acid was determined for the 0 to 1½ and the 1½ to 3 hour post-injection periods and it will be noted that the excretion after probenecid was less than when sodium pantothenate was given alone. With these indications that there was tubular secretion of pantothenic acid that could be inhibited by probenecid at these levels of plasma pantothenate, it seemed purposeful to perform formal clearance studies.

Clearance Studies. In one study simultaneous creatinine, pantothenate and PAH clearances were measured. PAH was given in an amount suitable for determining renal plasma flow. Patient R.R. fasted from midnight until 8 a.m., when she received 350 cc of water and Nembutal 1.5 grains. The patient was catheterized at 9:15 and a 20-minute control urine specimen was collected. Following this, a control blood sample was drawn and a priming injection of 500 mg of sodium pantothenate given. At the same time a sustaining infusion of sodium pantothenate and PAH in 5% dextrose in water was begun and a period of 46 minutes was allowed for equilibration. Throughout the study, pantothenate was infused at a rate of 0.157 mg/kg/min. and PAH at a rate of 0.09 mg/kg/min. Six blood specimens were obtained at appropriate intervals during the control and post-probenecid periods to allow construction of a curve for plasma levels from which the plasma values for each period might be calculated. After 3 control urinary collection periods a 2.0 g dose of probenecid was administered intravenously and 1.0 g was added to the infusion fluid. After 10 minutes was allowed for the establishment of the probenecid effect, urine was collected for 3 additional periods. Fig. 2 presents the observations that were made. In the control periods pantothenate clearance significantly exceeded creatinine clearance but did not approach PAH clearance. Under the influence of probenecid, pantothenate clearance approximated glomerular filtration rate. Probenecid also produced a significant diminution in PAH clearance but this clearance was not depressed to glomerular filtration rate. This degree of effect upon PAH clearance is in accord with that which we have described elsewhere(3)

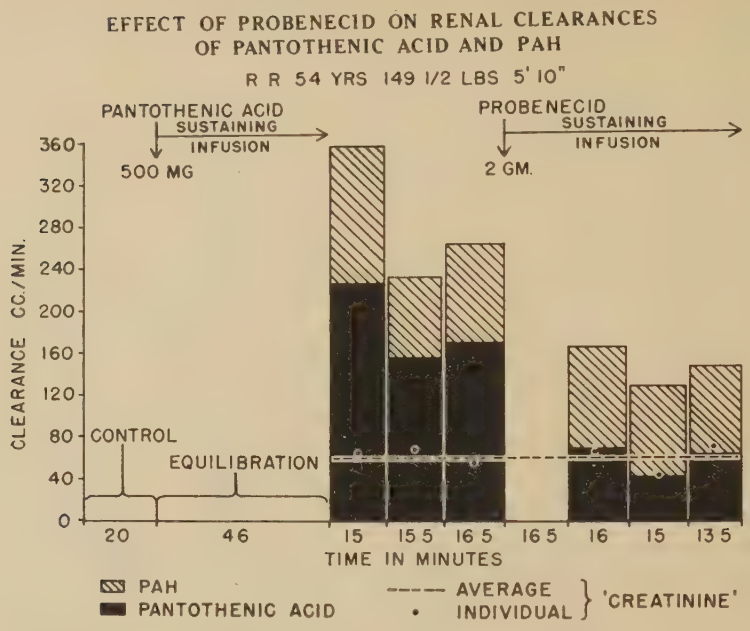

FIG. 2. Simultaneous measurement showed the normal pantothenate clearance to be intermediate between the PAH clearance and the creatinine clearance (glomerular filtration) rate. Under the influence of intravenously administered probenecid the clearance of pantothenate was depressed to creatinine clearance whereas PAH clearance was depressed only moderately.

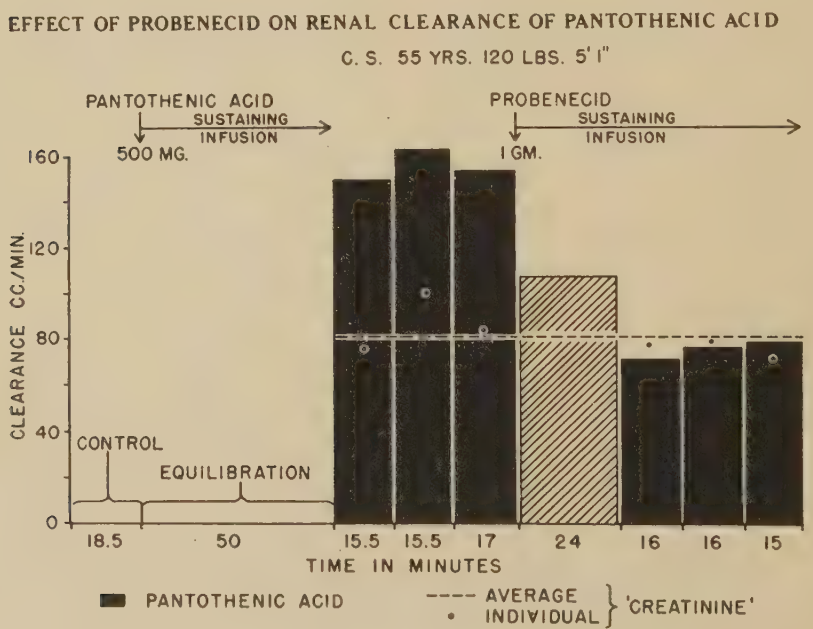

FIG. 3. Pantothenate renal clearance was measured in 3 control periods and found to exceed creatinine clearance (glomerular filtration) rate. Under the influence of intravenously administered probenecid the pantothenate clearance was depressed rapidly (within 24 min.) to a value approximating creatinine clearance rate.

and which has been confirmed by others(4). The probenecid plasma levels of 16 to 20 mg % that were maintained during this study were in excess of those required for maximum inhibition of tubular secretion of penicillin(2).

From this study it seemed reasonable to infer that pantothenic acid and PAH were probably handled by the same renal transport mechanism. Although it seemed unlikely that the small amounts of PAH administered to patient R.R. would significantly affect the pantothenate clearance, additional clearance studies were done in which the measurement of renal plasma flow with PAH was omitted. The interrelationship between substances being cleared has been previously noted(13).

The clearance study on patient C.S. was similar to that just described, the exceptions being the omission of PAH and the giving of a 1 g priming infusion of probenecid rather than 2 g. The same priming dose (500 mg) and the same rate of infusion of sodium pantothenate (0.157 mg/kg/min.) was employed.

Again (Fig. 3) the clearance of pantothenate was significantly greater than creatinine clearance during the control period but approximated creatinine clearance after probenecid. The hatched area represents the clearance for the post-probenecid equilibration period and indicates the rapidity with which probenecid exerts its effect.

Discussion. The studies of the renal clearance of pantothenic acid in dogs(7) demonstrated that the major portion, perhaps all, of the pantothenic acid present in normal plasma is in a nondiffusible form, and at low plasma concentrations this may complicate the calculation of clearance values. It was shown also that, when the plasma pantothenate level was elevated by intravenous pantothenate infusion, the added pantothenate was readily diffusible and was filtered at the glomerulus. Our patients had duplicate control values for plasma pantothenate that averaged 0.12 μg/cc (range of 0.08 to 0.21 μg). Pantothenate levels were not corrected by subtracting these control values for the calculation of pantothenate clearances. Even if the control pantothenate levels represented only nondiffusible material, as in the dog, the maximum error introduced would approximate 0.3%, since plasma concentrations of 30 to 50 μg/cc were maintained during these studies. We did not have available the technics for the study of the diffusibility of pantothenate when infused into man and our conclusions are drawn on the assumption that the findings of such a study would not alter the results qualitatively.

The foregoing observations indicate that pantothenic acid is another organic acid that can be actively secreted by the human renal tubule. Further, it seems likely that this is accomplished by the same renal transport mechanism that is involved in the tubular secretion of penicillin, PSP and PAH. Although it has not been demonstrated that any of these three latter substances will competitively inhibit the tubular secretion of pantothenate, it would be anticipated that PAH in quantity might do so in the same manner as it inhibits penicillin secretion(14). Two agents, carinamide and probenecid, have exhibited sufficient affinity for the transport system involved to inhibit reversibly the tubular secretion of all four of these reference substances. Carinamide was a safe and effective drug(15), but was impractical because of the large daily dose (18 to 24 g). Probenecid with a much greater affinity for the system involved (as judged by the smaller effective daily dose of 2 g) has been applied to the handling of a number of clinical circumstances.

The observation that probenecid will inhibit the tubular secretion of pantothenic acid has been made. The therapeutic implications of this observation are not apparent at this time.

Summary. 1. At plasma levels of 30 to 50 μg/cc, significant renal tubular secretion of pantothenic acid occurs. 2. It is probable that pantothenic acid is secreted by the same renal tubular transport mechanism involved in the transport of penicillin, phenolsulfonphthalein and PAH. 3. Probenecid can inhibit effectively the renal tubular secretion of pantothenic acid in man. 4. Probenecid enhances the plasma pantothenate levels obtained by the intravenous administration of sodium pantothenate in doses of 500 and 1000 mg.

We are indebted to Mr. P. W. Wilcox, Sharp and Dohme, Inc. for supplying us with intravenous preparations of sodium pantothenate and probenecid; to Dr. John Baer for the creatinine and PAH de-

terminations, and to Mrs. Charlotte A. Driscoll for the pantothenate assays.

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Conversion of Adrenocortical Hormones to More Polar Compounds by Adrenal Slices.* (20192)

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During the course of studies involving adrenal slice incubations, it was noted that extracts prepared from the slices after incubation sometimes contained 3 compounds which migrated very slowly on paper chromatograms. At other times these would not be present, or if present, in amounts which could not be detected.

The possibility was considered that these might represent conversion products of one of the known steroids. Consequently, hydrocortisone was incubated with adrenal slices. The incubation was carried out with 2.2 g of adrenal cortical tissue slices. Four and seven tenths mg of hydrocortisone were added to the incubating medium which was saline-phosphate (4:1), pH 7.3. Extracts made from the medium and slices were purified by silica gel chromatography. The purified fractions were then chromatographed on filter paper using the technic of Bush(1). In this particular incubation, adrenal slices incubated without added hydrocortisone produced no detectable quantities of the polar compounds, whereas the extracts from the incubation with added hydrocortisone showed the 3 polar compounds on the paper chromatograms (Fig. 1). The hydrocortisone had previously been shown to be chromatographically homogeneous.

The three compounds absorbed ultraviolet light. They reduced alkaline silver diamine and blue tetrazolium dye. They formed orange derivatives of dinitrophenyl hydrazine. The characteristics of these compounds suggest they may be identical with the three compounds "Ai", "Aii", and "Aiii", described by Bush(1) as appearing in the "amorphous" fraction of adrenal extracts.

Incubation of corticosterone with adrenal

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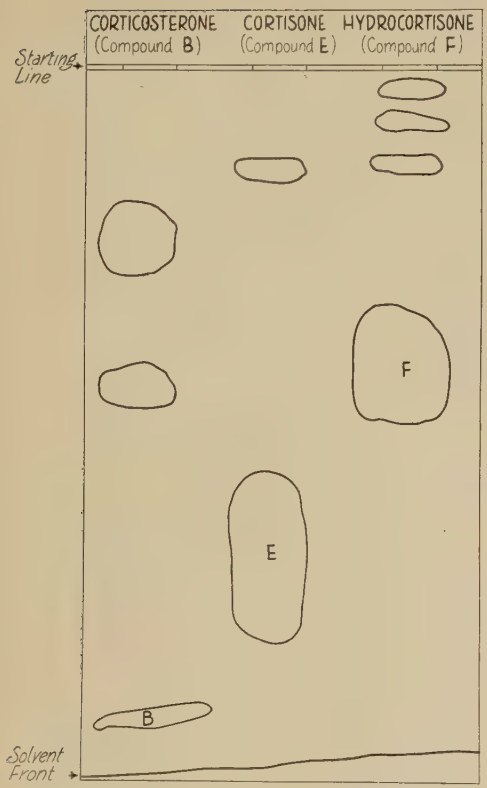

FIG. 1. Paper chromatogram of adrenal slice incubations of corticosterone, cortisone, and hydrocortisone. Compounds were outlined by their U. V. absorption. These substances also reduced blue tetrazolium.

tissue produced two compounds more polar than the starting material. A polar compound was produced when cortisone was incubated with adrenal tissue whose chromatographic mobility was similar to one of the compounds from the hydrocortisone incubation (Fig. 1).

Since the 3 products of hydrocortisone incubations possess the $\triangle^4$-3-ketone and α-ketol groupings and have distinctly lower mobilities than the original substrate, it is likely that additional oxygen functions have been put into the nucleus perhaps at carbons 6, 16, or both.

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Effects of Amphetamine and Its Isomers on Excretion of Sodium and Water in the Rat.* (20193)

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Recent reports(1-3) indicate that certain sympathomimetic drugs have important effects on the renal excretion of electrolytes and water. Although amphetamine is sympathomimetic, it has been reported that its urinary effects are variable(4) or negligible(5). The determination of urinary sodium has not, however, been carried out in animals or men receiving amphetamine; and in the experiments cited, no control of water or sodium intake was attempted. In the present communication, the effects of amphetamine and its isomers on the excretion of sodium and water in rats under controlled conditions of water and salt loading are described. Amphetamine (Benzedrine) and its dextrorotatory isomer (Dexedrine) and to a somewhat lesser extent the levorotatory isomer (Levedrine) appear to be powerful natriuretic and diuretic agents in all conditions studied.

Methods. Rats weighing from 200 to 350 g and of either sex were used. The drug to be investigated was given by intraperitoneal injection. Animals under salt load received 20 ml of isotonic saline by the same route. Other animals were used after an 18-hour fast during

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TABLE I. Sodium and Weight Losses in Rats Receiving Amphetamine and Its Isomers.

Drug	Dosage, mg/rat	State of animal	No. of animals	Duration metabolic period, hr	Sodium loss, mg/rat (range), ± = S.D.	Wt loss, g/rat (range), ± = S.D.
			(a)			
d-a*	1	18 hr fast	6	3	2.1 (1.9 – 2.3)	4.5 (4.0 – 5.0)
C	0	"	6	3	.25(.11– .39)	.5 (.33– .67)
			(b)			
d-a	1	Normal, unfasted	6	3	7.5 (4.3 –10.7)	12.0 (9.3 –14.7)
C	0	"	6	3	.8 (.5 – 1.3)	2.5 (2.0 – 3.0)
			(c)			
dl-a	1	20 ml isotonic NaCl	20	6	41.5 ± 2.2	16.9 ± .9
d-a	1	"	20	6	39.3 ± 4.5	15.7 ± 1.5
l-a	1	"	20	6	33.0 ± 2.2	12.8 ± .8
C	0	"	20	6	20.6 ± 3.5	8.4 ± .8
			(d)			
d-a	1	"	12	6	45.8 (42.1–47.3)	16.0 (13.5–19.5)
C	0	"	12	6	24.0 (22.6–27.1)	10.6 (9.2–11.9)
d-a	1	"	12	9	60.0 (50.0–68.2)	19.0 (15.3–23.1)
C	0	"	12	9	41.9 (38.3–45.1)	18.0 (14.1–22.5)
d-a	1	"	12	12	62.8 (59.2–66.0)	24.3 (22.5–26.5)
C	0	"	12	12	48.3 (47.0–49.5)	26.0 (23.5–28.2)
d-a	1	"	12	24	71.3 (65.8–77.0)	34.0 (29.6–38.3)
C	0	"	12	24	61.9 (57.0–68.2)	33.3 (30.0–36.4)
			(e)			
C	0	"	12	6	24.9 (20.7–29.2)	
d-a	0.1	"	12	6	24.6 (21.0–28.5)	
"	0.2	"	12	6	25.0 (20.5–28.4)	
"	0.5	"	12	6	28.6 (20.1–40.5)	
"	1.0	"	12	6	37.5 (34.0–42.8)	
"	2.0	"	12	6	35.0 (29.0–40.7)	

* a = amphetamine; C = control.

which no water was supplied. In some experiments animals were used without preliminary fasting or dehydration. The animals were placed in metabolic cages in groups of 3 to 5 and followed for metabolic periods of up to 24 hours. No food or water were allowed during the metabolic period. Sodium losses were determined by extracting the combined excreta with water and analyzing the extract with a flame photometer. Weight losses were used as a rough index of water losses. So far as possible, each rat was made to serve as its own control in consecutive experiments. Simultaneous control groups were also run in each experiment.

Results. Effect of d-amphetamine on sodium and weight losses in starving rats. Rats were starved for 18 hours and then received 1.0 mg of d-amphetamine in 1.0 ml of saline. Control animals received 1.0 ml of saline. The animals receiving d-amphetamine showed a considerable increase over the controls in both sodium excretion and weight loss during a 3-hour metabolic period. The results of a typical experiment are shown in Table I (a).

Effect of d-amphetamine on sodium and weight losses in unstarved rats. Results similar to those given above were obtained when there was no preliminary fast. The sodium and weight losses provoked by d-amphetamine were somewhat greater in these animals than in the starved animals. The results of a typical experiment are given in Table I (b).

Effects of amphetamine, d-amphetamine, and l-amphetamine on sodium and weight losses in salt loaded rats. In these experiments the control animals received isotonic saline intraperitoneally in 20 ml doses. The experimental animals received 1 ml of the drug alone with the saline. At the 6-hour period the results obtained were those presented in Table I (c). Similar results were obtained at 9, 12, and 24 hours, so far as sodium losses were concerned; weight losses tended to become equal in the drug treated and the control groups with time (Table I (d).)

Effect of dosage. The relationship between

dosage of d-amphetamine and sodium and weight loss was followed in salt loaded rats at periods of 3 to 24 hours. The results at 6 hours are typical of the findings at all periods. Doses of .1 and .2 mg per rat were completely ineffective so far as sodium and water losses were concerned; a very slight effect was observed at the .5 mg level; maximum effectiveness was attained at a dose level of 1.0 mg per rat and increasing the dose to 2.0 mg was without further effect. The results of an experiment with varying doses of d-amphetamine at the 6-hour period are given in Table I (e).

Effect of d-amphetamine on the glomerular filtration rate of fasting rats. The glomerular filtration rate was measured in 9 normal rats and 9 d-amphetamine treated rats by the "undisturbed" method of Pultz, Herrold, and Sapirstein(6). The animals were not under saline load and filtration rates were measured after an 18-hour fast. In preliminary experiments on nephrectomized rats, the "effective delay time" was found to be unchanged by the administration of 1.0 mg of d-amphetamine. The volume of distribution of mannitol was likewise unchanged from the normal. (Effective delay time normals, 3 minutes; d-amphetamine treatment, 3 minutes; mannitol space, nephrectomized normal, 28.6% body weight, d-amphetamine treatment, 28.5% body weight). The mannitol clearance in the normal animals was found to be .62 ml/100 g/min. (S.D. .08) and in the d-amphetamine treated animals .85 ml/100 g/min. (S.D. .06).

Discussion. In all experiments animals receiving amphetamine or either of its isomers showed evidence of central nervous system stimulation and increased activity. This occurred even at the lower dosage levels used in the experiments described in Table I (e), which were without effect on sodium or weight losses. From these findings, it appears improbable that the effect on sodium and weight losses can be attributed to increase somatic activity.

The effects of amphetamine and d-amphetamine appear to be quantitatively comparable. l-amphetamine appears to be somewhat less active than d-amphetamine, but nevertheless possesses considerable activity. The fact that the racemic mixture of d- and l-amphetamine, which would be expected to be less active than d-amphetamine, was actually as active as d-amphetamine can probably be attributed to the use of a supramaximal dose of the mixture.

The increased elimination of sodium and water (measured as weight loss) which are observed in the drug treated animals can be accounted for readily by the increase in the glomerular filtration rate. In the fasted animal this increase in filtration rates amounts to .23 ml/100 g/min. or to 35.5 ml/rat/hr. In terms of sodium this is equivalent to 128 mg/rat/hr. In the 3-hour metabolic period, it is necessary to account for only 4.0 ml of water and 1.85 mg of sodium excess in the drug treated fasting animals. Clearly, there is no need to postulate a decrease in tubular reabsorption of sodium and water after drug treatment. In view of the sympathomimetic properties of amphetamine, it is of interest that similar changes in filtration rate have been reported in rats treated with noradrenaline by Eversole *et al.*(1); these authors, however, interpreted the fact that creatinine U/P ratios were altered more than filtration rates to indicate that there was decreased tubular reabsorption of sodium and water with noradrenaline; this argument does not appear to be valid.

Insufficient information is available from the present experiments to indicate definitely whether the increased filtration rate which we believe to be responsible for the increased elimination of water and sodium is due to intrarenal hemodynamic alteration or is consequent on a general rise in the arterial pressure. However, in preliminary experiments in human subjects, we have noted increased sodium and water excretion without blood pressure rise when d-amphetamine is taken. It appears probable that the effect of amphetamine on the renal circulation is a direct one, consisting of afferent dilatation, efferent constriction, or both.

The early rapid weight loss sometimes observed in human subjects on controlled diets who are receiving d-amphetamine may be a consequence of a sodium and water wasting effect similar to what has been reported here in rats. In prolonged therapy, however, there

seems to be no doubt that the weight loss observed is due to diminished food intake.

Although the salt wasting effects of amphetamine and d-amphetamine in the animal loaded with isotonic saline requires dosage levels in the rat far above those which would be considered therapeutic in man, we have found in the preliminary experiments quoted above that fasting human subjects show considerable natriuresis and diuresis with doses of 5.0-10.0 mg of d-amphetamine. The possibility that amphetamine and other sympathomimetic agents may be of value as adjuncts in the treatment of edema is being investigated.

Summary and conclusions. 1. Amphetamine and its isomers are diuretic and natriuretic in starved, normal and salt loaded rats. 2. The glomerular filtration rate of starved rats is elevated by d-amphetamine. The elevation of filtration rate is sufficient to account for the diuresis and natriuresis observed without postulating a decrease in tubular absorption of water or sodium.

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In vitro Inactivation of Newcastle Disease Virus by Penicillin Preparations. (20194)

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In the course of experiments in which it was attempted to estimate the concentration of Newcastle disease virus (NDV) by titration in embryonated eggs, a delay of the death of infected embryos was observed when the infecting virus suspension contained penicillin. Incubation of suspensions containing up to 10000 ELD_{50} (lethal for embryos) of the virus with 5000 units of penicillin for a few hours before injection into chick embryos resulted in an apparent 100-fold decrease in the infectivity of the virus. Streptomycin at concentrations up to 5 mg/ml had no detectable influence on the virus.

The present communication reports on the action of penicillin on NDV.

Materials and methods. Virus. The Newcastle disease virus used in these experiments was isolated from an epidemic in 1949† and stored in the form of infected allantoic fluid at –25°C for approximately 2 years. The virus killed susceptible chickens within 6 days after inoculation, and embryonated eggs within 72-96 hrs. In some experiments the live virus vaccine of Komarov and Goldsmit(9) was used. Titrations were carried out in 9-12 days embryonated eggs. Serial 10-fold dilutions of virus were prepared and 0.1 ml of each dilution was inoculated in the allantoic sacs of each of 4 to 6 eggs. Infected eggs were candled daily. Embryos dying within 24 hours of incubation were discarded. All eggs showing death of embryos after 24 hours were chilled for a few hours, opened, and their allantoic fluid tested for the presence of virus by means of a plate hemagglutination test (13). Virus titers were estimated according to the method of Reed and Muench(18). *Penicillin.* Crystalline penicillin G was used throughout (sodium salt—Merck, or potas-

* The author is indebted to Dr. J. Mager for helpful criticism.

† Kindly supplied by Dr. Bornstein of the Government Veterinary Institute.

sium salt—Merck or Pfizer), showing an activity of 1500 to 1666 units per mg. The effectiveness of various means of inactivation of penicillin—such as heating, alkali, acid or penicillinase treatment—was also measured by means of dilution method against *Staphylococcus aureus*. *Penicillinase* (Wellcome) of the potency of 100000 penicillin neutralizing units per ml with 0.25% phenol preservative was used in certain experiments to inactivate the penicillin. Control tests showed that under the conditions of the experiment, neither the penicillinase (fresh as well as autoclaved) nor the phenol, exerted any measurable effect on the embryos or the virus.

Results. I. *Factors influencing action of penicillin on NDV.* A. *Time of incubation of penicillin—NDV mixtures.* Infected allantoic fluid containing 10^8-10^9 ELD_{50} per 0.1 ml was diluted 100-fold in sterile saline or phosphate buffer, in order to exclude possible interference by the concentrated allantoic fluid. The diluted virus (5 ml) was mixed with penicillin and incubated at 37°C for various periods of time. At the end of incubation period, the virus-penicillin mixture was titrated by inoculation of 10-fold dilutions into embryonated eggs. In this procedure the quantity of penicillin injected into the egg was less than 1000 units. At a concentration of 2000-5000 penicillin units per ml, the reduction of infectivity of the virus was not apparent within one hour of incubation, but became very pronounced by the third hour of incubation, by which time up to 10^5 ELD_{50} of the virus had been inactivated. As the concentration of penicillin was increased (up to 50000 units per ml) there was a corresponding decrease in the incubation time needed for significant inactivation of the virus. In the presence of concentrations higher than 100000 units per ml the virus was inactivated without prior incubation. It should be mentioned that the hemagglutinating titer of the virus was not affected by penicillin.

B. *Concentration of penicillin.* When NDV was incubated for 3 hours with penicillin at concentrations ranging from 200 to 20000 units per ml and the residual virus titrated by inoculation of serial tenfold dilutions of the mixture (in eggs), the degree of inactivation of the virus was found to be proportional to the concentration of the antibiotic (Fig. 1). At a concentration of 100-1000 units per ml the action of penicillin on the infectivity of the virus was indiscernible, but at 20000 units per ml a 10000-fold reduction of the virus concentration was observed. In several experiments 10000 units per ml inactivated completely 2×10^6 ELD_{50} of the virus.

II. *Activity of penicillin against NDV in vivo.* Serial 10-fold dilutions of NDV were prepared. From each dilution one ml was removed and mixed with one ml of penicillin solution containing 500000 units per ml. Two-tenths of the resulting mixtures were immediately injected into embryonated eggs and 10-day-old chicks. This procedure resulted in the protection of embryonated eggs and chicks against up to 4000 ELD_{50} of the virus. In the presence of higher concentrations of penicillin (800000 units per ml) even 10^6 ELD_{50} were not lethal to the embryos and no virus could be detected in them. The protection was, however, elicited only when the antibiotic and the virus were mixed before injection. Injection of penicillin prior to, or subsequent to the virus, protected neither chick embryos nor chicks. Simultaneous injection of virus and penicillin at different sites in chicks did not protect them against the disease. Prophylactic treatment by repeated injections of penicillin into birds exposed to an infected flock did not prevent them from contracting the disease at the same rate as non-treated chicks.

III. *Effect of various treatments on antiviral activity of penicillin.* *A. Activity of heat-treated penicillin.* When a solution of penicillin containing 10000 or 100000 units per ml was immersed for 10-15 minutes in a bath of boiling water, its antistaphylococcal activity fell to 1-100 units per ml. However, the infectivity of NDV incubated for 3 hours with this heated penicillin was reduced to the same extent, as when incubated with unheated penicillin (Table I). The virus was not affected whatsoever, when incubated with a quantity of fresh penicillin, which showed the same antistaphylococcal activity as that, which remained after heat inactivation.

B. *Activity of acid—or alkali-treated penicillin.* Five ml of a solution containing 50000

TABLE I. Comparison of Action of Fresh and Heated Penicillin on NDV.

NDV virus batch	Penicillin*	Penicillin conc., units/ml (×1000)	Incubation Time, hr	Incubation Temp., °C	Difference in virus conc. (log ELD_{50}) before and after incubation: Virus alone	Virus treated with: Fresh penicillin	Virus treated with: Heat inactivated penicillin
5	M-S	10	3	37	.0	3.5	2.0†
5	"	10	3	37		5.3‡	4.8
9	R-K	8	1†	45	.25	6.2‡	5.7†
			3	37			
9	"	8	3	37	.0	4.6	3.0
9	"	10	3	37	.7	2.1	4.4
10	"	10	3	37	.1	2.1	2.5‡

* M-S = Sodium G Penicillin (Merck) 1666 units/mg; R-K = Potassium G Penicillin (Rafa, Jerusalem) 1500 units/mg.

† Reduction of activity greater than indicated in table (titration endpoint not reached).

‡ *All* virus activity destroyed.

TABLE II. Comparison of Action of Penicillinase Treated and Untreated Penicillin on Newcastle Disease Virus.

Virus batch	Penicillin*	Penicillin conc., units/ml (×1000)	Virus conc. (log ELD_{50}): Virus alone	Virus + penicillin	Virus + penicillin + penicillinase	Reduction of virus activity by penicillin (log ELD_{50})	% inactivation of penicillin by penicillinase‡
A							
1	M-S	6	5.6	4.3	5.5	1.3	
5	"	10	5.0	2.5	3.5	2.5	90
9§	R-K	8	6.6	5.4	6.3	1.2	87
9§	"	8	6.6	2.0	6.0	4.6	99.99
9	"	10	6.6	4.5	5.3	2.0	84
10	"	10	2.5	.37	.5	2.13	33
B							
9	HR-K	8	6.6	3.5	5.8	3.0	99.4
9	"	10	6.6	2.3	4.6	4.3	99.5
10	"	10	2.5	0 †	1.7	2.5	98
C							
13	M-S	20	5.3	3.3	4.1	2.0	84
					5.1‖		98.4
14	"	20	5.4	.5	3.1	4.9	99.76
					5.2‖		100
13	"	20	5.0	0	0	5.0†	0
					5.5‖		100

* and † as in Table I. HR-K = Heat-inactivated potassium G penicillin.

‡ $\left(100 - \frac{\text{antilog column VI} \times 100}{\text{antilog column VII}}\right)$ %.

§ Data represent results after incubation of virus + penicillin mixture 1.5 and 3 hr respectively.

‖ Penicillin incubated with *autoclaved* penicillinase 30 min. before addition of virus; additional incubation 3 hr.

units of penicillin in distilled water or saline were treated wth 0.1 ml of N/1 hydrochloric acid or with 0.1 ml of N/1 sodium hydroxide and incubated for 30-60 minutes at 56°C. The acid treatment presumably brings about transformation of penicillin to penillic and penilloic acid, whereas alkali causes the formation of penicillic acid in a manner similar to the action of penicillinase(4). At the end of the incubation period the solutions were neutralized and incubated with NDV for 3 hrs at 37°C. Both alkali and acid treatment reduced the antistaphylococcal potency of penicillin 100-1000-fold, but whereas the acid treated penicillin lost completely its virucidal property (irrespective of its antistaphylococ-

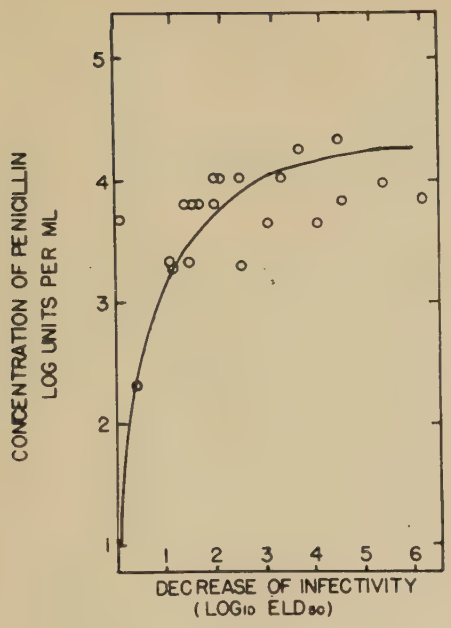

FIG. 1. Reduction of infectivity of NDV by penicillin (incubation 3 hr).

cal potency), the antiviral activity of the alkali treated antibiotic was reduced at the same rate as the antistaphylococcal activity.

C. *Influence of penicillinase on the antiviral activity of penicillin.* When penicillinase‡ at adequate concentration was mixed with penicillin *prior to* incubation of the latter with the virus, the inactivation of the virus was prevented (Table 2A). Penicillinase also inhibited the antiviral activity of heat treated penicillin (Table IIB). Later experiments showed that autoclaved, *i.e.* enzymatically inactive penicillinase also neutralized the antiviral activity of fresh penicillin (Table IIC). When penicillinase was added to the mixture of virus and penicillin *at the end* of the incubation period, no reversal of virus inactivation took place.

IV. *Activity of penicillin against other viruses.* Preliminary experiments showed that penicillin was active against the viruses of influenza (PR8) and Eastern Equine Encephalitis (EEE). The incubation of these viruses in 5 ml saline with 50000 units per ml of penicillin G for 3 hours resulted in a 10000-fold reduction of influenza activity and complete destruction of 10^8 LD_{50} of EEE.

Discussion. It is generally assumed that penicillin does not affect microorganisms which have no measurable activity outside the host cell. Even the large viruses like Rickettsiae and members of the Psittacosis-Lymphogranuloma group that are inactivated *in vivo* (embryonated eggs and mice) by relatively low doses of penicillin, are affected *in vitro* only by very high concentrations (40000-100000 units/ml) of this antibiotic(5,7,16). Eaton *et al.*(2) and Hamre and Rake(7) present evidence that the effect of penicillin *in vitro* on agents of lymphogranuloma venereum, feline and murine pneumonitis might have been due to impurities. It was also found by Groupé and Rake(6) that a certain batch of commercial penicillin, with a potency of 988 units/mg, destroyed the viruses of canary pox, smallpox and vaccinia after contact of 2 hours. Inactivation by clarase or by boiling did not affect the antiviral property of this penicillin; crystalline penicillin G (25000 units) alone was inactive against these viruses. The effect was apparently due to impurities. The results obtained by us with crystalline penicillin G indicate that we are not dealing with the same active entity as Groupé and Rake, though the possibility that impurities may be responsible for the findings cannot be ruled out. Penicillin has been reported to be inactive against the viruses of vaccinia(2,8,16), fowl-pox(20), rabies(11,19), herpes and influenza(21), mumps(1), eastern equine and St. Louis encephalitis(16), coxsackie(14), sarcoma(11), bacterial viruses(15,17) and plant viruses(13). In most of these investigations (1,8,10,11,16,17,20,21), however, the concentration of penicillin was comparatively low and/or no incubation *in vitro* with the virus was attempted.

The phenomenon of inactivation of NDV by penicillin is surprising, for this virus is a member of a group which was hitherto thought to be refractory to penicillin. The lack of therapeutic activity of penicillin against NDV *in vivo* (II) may perhaps be explained as being due to insufficient concentrations of the antibiotic attained in the chick embryos or chicks. The maximal concentration of peni-

‡ The term penicillinase refers to the commercial preparation described in *Methods*.

cillin in the egg or chick could not have exceeded 1000 units/ml, which was found to be insufficient for the inactivation of the virus *in vitro*.

The inability of the penicillinase preparation to restore the virus to its original activity, when added to the penicillin-virus mixture at the end of the incubation period, indicates an irreversible inactivation of the virus. Whilst acid or alkali treatment destroys both the antiviral and the antistaphylococcal properties of penicillin, boiling or treatment with autoclaved penicillinase affect these properties in a different manner: Heat treated penicillin has lost its antistaphylococcal activity, but is still fully active against the virus; a heat-stable, non-enzymatic factor present in the penicillinase abolishes the antiviral, but not the antibacterial activity of penicillin.

The problems which remain to be elucidated are: 1) whether the antiviral properties of "penicillin" are due to a) some thermostable, functional group of the penicillin molecule itself, or b) a highly active, contaminating substance present in crystalline penicillin G which is heat stable but sensitive to acid and alkali; 2) what is the heat-stable substance in penicillinase which destroys the antiviral activity of penicillin.

Summary. 1. When Newcastle disease virus is mixed with penicillin, inactivation of the virus takes place. The extent of the inactivation depends both on the concentration of penicillin and the time of incubation of the mixture. Incubation of the virus *in vitro* for 3 hours with more than 5000 units per ml of crystalline penicillin G results in at least a 100-fold reduction of the infectivity of the virus and up to 2 million ELD_{50} of the virus may be completely inactivated. 2. The virus inactivating property of penicillin may be destroyed by acid or alkali treatment but is not affected by heating. Penicillinase also inactivates the antiviral properties of fresh and heated penicillin. However, this effect is apparently not due to enzymic action. Virus *previously* inactivated by fresh or heated penicillin cannot be restored to its original potency when penicillin is neutralized by penicillinase. 3. Injection of 50000 units of penicillin *mixed* with the virus protects chick embryos and 10-day-old chicks against as many as 4000 ELD_{50} of the virus. At this concentration, however, penicillin has no prophylactic or therapeutic activity.

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Thiamine Sparing Action of Ascorbic Acid on *Lactobacillus Fermenti* 36.* (20195)

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The stimulatory effect of ascorbic acid on lactic acid bacteria has recently been demonstrated. Kitay *et al.*(1) found that ascorbic acid was rarely active and only at higher concentrations. It promoted growth of *L. leichmanii* 313 and *L. acidophilus* 4913 but was inactive in promoting growth of *L. acidophilus* 832, *L. delbrueckii* 730, and *Leuconostoc citrovorum* in defined media. In the study on the growth of *L. casei*, Rickes *et al*(2) concluded that the effectiveness of ascorbic acid and d-isoascorbic acid in further increasing the rate of growth of this microorganism presumably was due to the reducing effect. Katznelson(3) found that sodium thiosulfate and ascorbic acid may partially replace the thiamine requirement of *Bacillus paraalvei*. Recently, using *L. fermenti* 36 in the microbiological assay of thiamine(4), we have noted that when as little as 0.4 mg of ascorbic acid was added to 10 ml of assay medium, the growth effect of thiamine was greatly enhanced. Furthermore, the organism responded quantitatively to ascorbic acid in a thiamine-free medium, reaching a maximum at 1 mg per tube. Since thiamine is intimately concerned with carbohydrate metabolism, especially with the degradation of pyruvic acid, the observation of the thiamine sparing action of ascorbic acid on growth of this microorganism throws interesting light on the role of ascorbic acid.

It is the purpose of this paper to attempt to clarify in part the interrelationship of these two vitamins.

Methods. *Lactobacillus fermenti* 36 was grown in the medium used for thiamine assay (5,6). Varying amounts of thiamine or ascorbic acid and other substances to be tested were measured into tubes, diluted to 5 ml, and 5 ml of medium added. Boiled alkali-digested peptone was used in the medium in order to secure a lower blank. The tubes were plugged, steamed and inoculated with a very dilute suspension of *L. fermenti* 36 inoculum. Growth was measured turbidimetrically after 16 to 24 hours of incubation with a Klett-Summerson photoelectric colorimeter, using a 54 filter. The results are expressed as Klett units.

Results. Stimulatory effect of ascorbic acid on growth of L. fermenti 36 supplied with suboptimum amounts of thiamine. Table I shows that the growth effect of thiamine was greatly enhanced when 2 mg or 5 mg of ascorbic acid were added either before steaming or aseptically after steaming. During the early growth period, thiamine will not prevent the stimulatory effect of ascorbic acid nor will ascorbic acid prevent the similar effect of thiamine.

Effect of thiamine and ascorbic acid on growth of L. fermenti 36. When graded amounts of ascorbic acid were added, either before steaming or aseptically to the thiamine-free medium, the early growth of *L. fermenti* 36 responded quantitatively. A typical set of data is shown in Table II. However, when the incubation period was prolonged to 72 hours thiamine promoted better growth and more acid production than did ascorbic acid. This indicates that ascorbic acid cannot completely replace thiamine in the growth of this microorganism. It suggests that ascorbic acid may permit the synthesis of thiamine to a limited degree or that ascorbic acid may partially perform the physiological role usually carried out by thiamine. When pyrithiamine was added to the medium this inhibitor was much more effective against ascorbic acid than against thiamine. Furthermore, much more pyrithiamine was required to have the same inhibitory effect when thiamine and ascorbic acid were present together than when they

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TABLE I. Stimulatory Effect of Ascorbic Acid on Growth of *Lactobacillus fermenti* 36 when Supplied with Suboptimum Amounts of Thiamine.

Thiamine level, γ	Turbidity reading: Ascorbic acid added before autoclaving		Ascorbic acid added aseptically	
	0	5 mg/tube	2 mg/tube	5 mg/tube	2 mg/tube
.00	27	67	69	53	58
.01	41	93	99	84	82
.02	48	102	111	99	100
.03	52	116	122	97	110
.05	55	132	133	114	113

Turbidities were measured with a Klett-Summerson photoelectric colorimeter, using a 54 filter, after 17 hr incubation at 37°C and expressed as "Klett Units." Assay medium described in (4) was used.

TABLE II. Effects of Thiamine and Ascorbic Acid on Growth of *Lactobacillus fermenti* 36.

Thiamine, γ (only)	Turbidity reading	Ascorbic acid, mg (only)	Turbidity reading: Added before autoclaving	Added aseptically
.00	27	.0	26	27
.01	41	.5	47	51
.02	48	1.0	62	54
.03	52	2.0	70	54
.05	55	5.0	68	52

Turbidities measured with a Klett-Summerson photoelectric colorimeter, using a 54 filter, after 17 hr incubation at 37°C and expressed as "Klett Units." Assay medium described in (4) was used.

were used separately. These data as recorded in Table III suggest the synergistic effect of ascorbic asid on thiamine activity.

In studying the mechanism of the growth-promoting effect of ascorbic acid on *L. leichmanii,* Welch *et al.*(7) suggested that the apparent microbiological activity of ascorbic acid may be attributed to the conversion of oxidation products of vit. B_{12} to microbiologically more active forms. It seems unlikely that a similar role for ascorbic acid would apply in this case, because if the assay medium contains some oxidized form of thiamine, then addition of ascorbic acid, which would convert the oxidized product back into thiamine, should not further enhance the growth-promoting activity of thiamine as the latter was present in sufficient quantity in the assay medium. Stokstad *et al.*(8) reported that thioglycolic acid and ascorbic acid protected vit. B_{12} against destruction during autoclaving of assay media containing the vitamin. Our results show that when the ascorbic acid was added aseptically (sterilized by filtration) to the medium after steaming it also enhanced the growth-promoting activity of thiamine. Therefore, this would seem to indicate that ascorbic acid must have a special function on the carbohydrate metabolism. In a separate experiment, either thioglycolic acid or

TABLE III. Inhibition Effect of Pyrithiamine on Utilization of Thiamine or Ascorbic Acid for Growth of *L. fermenti* 36.

Pyrithiamine added/tube, γ	Turbidity reading: Thiamine level, γ 0	0.05	0.1	Pyrithiamine added/tube, γ	Turbidity reading: Ascorbic acid level, mg 0.5	1.0	1.5	Pyrithiamine added/tube, γ	Turbidity reading: 1 mg vit. C + 0.05 γ B_1	+ 0.1 γ B_1
0	19	96	116	0	47	54	55	0	114	133
.1	5	89	—	.01	5	6	38	.1	116	—
.2		68	—	.02	5	8	7	.2	97	—
.3		45	93	.03	5	5	6	.3	74	133
.4		25	72	.04	5	5	5	.4	54	132
.5		—	36					.5	—	109
.6			15					.6	—	89
Amt pyrithiamine required for 50% growth inhibition		.29	.44		<.01	<.01	.014		.38	.71

Incubation period 16 hr.

TABLE IV. Effect of Several Enzyme Inhibitors on Utilization of Thiamine or Ascorbic Acid for Growth of *L. fermenti* 36.

Substances added	Range used/tube	Thiamine level 1.0γ	Thiamine level 0.5γ	Ascorbic acid level 2.0mg	Ascorbic acid level 1.0mg
	mg	mg	mg	mg	mg
Iodoacetate	0– .4	.16*	.16	.40	.38
2,4-dinitro-phenol	0–2	.16		†	
KCN	0–2	†		†	
NaF	0–2	2.0		†	
Malonic acid	0–2	†		†	

Incubation period 16 hr.
* Amount of inhibitor necessary to cause 50% inhibition.
† No inhibition.

cysteine in an amount of 2 mg per 10 ml medium had shown to enhance the growth-promoting activity of thiamine by approximately 15%. However, these two compounds do not support growth of *L. fermenti* 36 without the presence of thiamine. This result further proves that the thiamine sparing action of ascorbic acid is not due to its reducing effect. When sodium ascorbate was added to the assay medium containing no glucose, no growth of this organism was observed even in the presence of thiamine indicating that ascorbic acid could not be used as substrate.

Effect of several metabolic inhibitors on Growth of L. fermenti 36 in presence of thiamine or ascorbic acid. In order to determine whether the thiamine and ascorbic acid have the same mechanism on the growth-promoting effect, several other known metabolic inhibitors were added individually to assay media which contained either thiamine or ascorbic acid. Increasing amounts (0-2 mg) of the inhibitors were added to 10 ml of the assay media. The growth of *L. fermenti* 36 was measured after 16 hours of incubation. The 50% inhibition indices of all metabolic inhibitors used were determined and are recorded in Table IV. Iodoacetate, in a concentration of 0.16 mg per 10 ml assay medium, was found to cause 50% inhibition of the growth promoted by either 1 γ or 0.5 γ of thiamine. Higher concentrations, 0.40 mg and 0.38 mg, were required respectively in the cases of ascorbic acid. With 2,4-dinitrophenol, 0.16 mg was required to inhibit 50% of the growth which was promoted by 1 γ of thiamine and no inhibition was observed when ascorbic acid was present. A difference was also noted between the growth-promoting effect of thiamine and ascorbic acid when sodium fluoride was used. These results all pointed to the fact that ascorbic acid must function in a different metabolic pathway when compared to thiamine. 2,4-dinitrophenol has been shown to retard pyruvic acid oxidation by brain tissue(9). Addition of fluoride presumably inhibited the breakdown of phosphoglyceric acid. In the growth of *L. fermenti* 36 which was promoted by ascorbic acid in the absence of thiamine no inhibition by fluoride was observed. This would indicate that oxidation of glucose by *L. fermenti* 36 may by-pass the step inhibited by fluoride when ascorbic acid is present.

Thiamine Synthesis of L. fermenti 36 Grown in Assay Medium Containing Thiamine, Ascorbic Acid or both. To one liter of assay medium(5), 10 γ of thiamine or 200 mg ascorbic acid were added; the medium was then sterilized by two 30-minute periods of steaming. Ten drops of concentrated inoculum of *L. fermenti* 36 were used and the flasks were incubated at 37°C for 18 hours. The cells were centrifuged, washed twice with distilled water, and finally dispersed in water and diluted to 50 ml. One-half (25 ml) of of this cell suspension was digested with a takadiastase and papain mixture to liberate the thiamine(5). Thiamine determination was performed microbiologically using *L. fermenti* 36 as a test organism. A modified(4,5) assay medium which contained an additional 20 mg maltose and 2 mg ascorbic acid per 10 ml assay tube was used. The other half of the cell suspension was dried in order to determine the amount of cell production.

The experiment was repeated 3 more times. In the second experiment the growth was found to be very light after 18 hours, so growth was allowed to continue for 44 hours. In the third and fourth experiments both thiamine and ascorbic acid were included in the assay medium. In the fourth experiment, the thiamine content of the cells was determined by the thiochrome method(10), as a further check that thiamine was the factor being measured. The results are shown in

TABLE V. Thiamine Synthesis of *L. fermenti* 36 Grown in Thiamine Assay Medium Containing Either Thiamine, Ascorbic Acid or Both.

	Substance added in thiamine assay medium					
	Thiamine, 10 γ/liter		Thiamine, 10 γ and ascorbic acid, 200 mg/liter		Ascorbic acid, 200 mg/liter	
Incubation, hr	Cells production, g/liter	B_1 content, γ/g	Cells production, g/liter	B_1 content, γ/g	Cells production, g/liter	B_1 content, γ/g
18	.580	12.8			.159	6.6
44	.345	9.6			.148	4.2
24	.720	11.8	1.020	13.5	.356	7.0
24*	.250	6.4	.366	6.9	.086	3.9

* *L. fermenti* 36 ATCC 9338 used in this experiment. Thiamine values determined by thiochrome method. Fluorescence measured with a Beckman DU spectrophotometer.

Table V. The thiamine content of *L. fermenti* 36 cells grown in a medium containing ascorbic acid was found to be about one-half of the amount present in the cells grown in the thiamine medium. Furthermore, when both thiamine and ascorbic acid were present in the assay medium, not only was more growth of *L. fermenti* 36 observed, but the amount of thiamine in the cell was found to be increased by 10 to 15% over the cells grown in the thiamine medium. From this result, it may be assumed that the stimulatory effect of ascorbic acid on growth of *L. fermenti* 36 may function in part by stimulating the synthesis of thiamine.

Summary. 1. Ascorbic acid has been found to have thiamine sparing action on the growth of *L. fermenti* 36. The addition of both ascorbic acid and thiamine gives a greater growth response than either one when added alone. 2. Addition of pyrithiamine inhibits the utilization of ascorbic acid for growth of *L. fermenti* 36 in a thiamine-free medium. This result shows that ascorbic acid may function in part by stimulating the synthesis of thiamine. 3. Addition of iodoacetate, 2,4-dinitrophenol or sodium fluoride to the thiamine assay medium containing either thiamine or ascorbic acid shows the difference in inhibition of growth of *L. fermenti* 36. These results indicate that ascorbic acid must also function in a different metabolic pathway as compared to thiamine in carbohydrate metabolism. 4. It was found that *L. fermenti* 36 was able to synthesize thiamine when ascorbic acid was present. With the presence of both ascorbic acid and thiamine in the assay medium, the cell was found to contain more thiamine than the cell grown in thiamine medium alone.

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Influence of Enteramine (5 Hydroxytryptamine) on Renal Function of the Dog. (20196)

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Among the hormonal factors controlling water excretion by the kidney, enteramine is to be listed. Enteramine is the specific hormonal product of the enterochromaffin cell system which is widely distributed in vertebrates and invertebrates(1). It was isolated from beef serum by Rapport *et al.*(2), and tentatively identified as 5-hydroxytryptamine and named serotonin. Enteramine was isolated from the extracts of *Octopus vulgaris* and *Discoglossus pictus* by Erspamer and Asero(3); it has been simultaneously and independently synthesized in 3 different laboratories(3-5,9). 5 hydroxytryptamine was said to be vasoconstrictor and pressor(2,6), general smooth muscle stimulator(7), to play an important role in hemostasis(8). Erspamer claims that the physiological and main activity of the substance is exerted on the kidney, where it promotes water retention by reducing the GFR through the increased tone of the glomerular afferent vascular bed: extrarenal vascular and spasmogenic effects are to be considered only as pharmacological actions(3,14).

The present study was undertaken to investigate the effect of different doses of enteramine on the urine volume and the renal function of the hydrated dog.

Methods. Assays on the antidiuretic activity of enteramine were performed on trained, unanaesthetised and colpotectomized female dogs averaging 15 kilos in weight. The dogs were maintained for 16-20 hours prior to the experiment without food but with free access to water. For oral hydration, 50 ml per kilo of tap water were administered by stomach tube half an hour before the start of the experiment; for intravenous hydration, distilled water or saline were infused at a constant rate of 3-5 ml per minute. During clearance periods, lasting 10-20 minutes, the dogs were restrained loosely on a comfortable animal table. Blood samples were collected midway in the periods, no correction being made for delay time. Catheterisation was performed with a rubber catheter and each period was terminated by a 20 ml saline washout. The clearance of sodium thiosulfate was used as a measure of filtration rate, a quantity of thiosulfate being infused necessary to sustain plasma levels over 20 mg %; the clearance of sodium-aminohyppurate was used as a measure of the renal plasma flow, the plasma levels of PAH being maintained between 1 and 3 mg %. Thiosulfate and PAH were determined in the urine and plasma according to Brun(10) and Finkelstein and Aliminosa(11), respectively. Enteramine picrate was dissolved into hot water and injected subcutaneously or, intravenously; the quantity used is expressed as picrate. The picrate contains 41% of the free base 5 hydroxytryptamine.

Results. The effect of enteramine was studied in a series of 30 dogs; the results of 3 representative experiments are summarized in Fig. 1, 2, 3.

Urine volume was reduced according with the pre-injection values; at initial rates of 7-9 ml per minute, doses of 0, 1-0, 3 mg per kilo of enteramine decreased the urine flow to 1-0, 8 ml per minute, while at initial values of 1-3 ml per minute, enteramine even in doses of 0, 5-1 mg per kilo was unable to reduce the

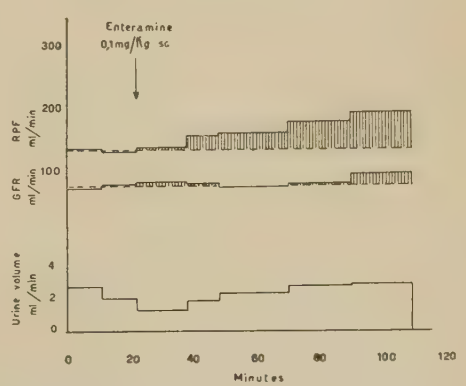

FIG. 1. Effect of 0,1 mg/kg of enteramine on urine volume, GFR and RPF of the hydrated dog.

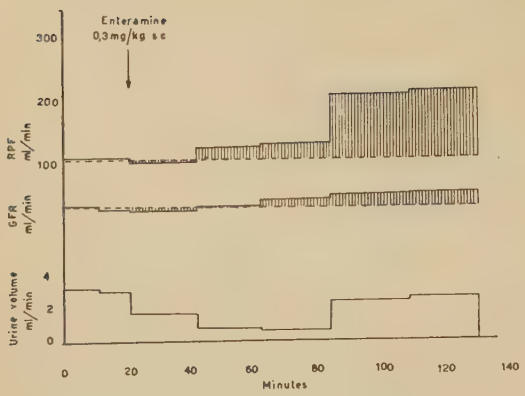

FIG. 2. Effect of 0,3 mg/kg of enteramine on urine volume, GFR and RPF of the hydrated dog.

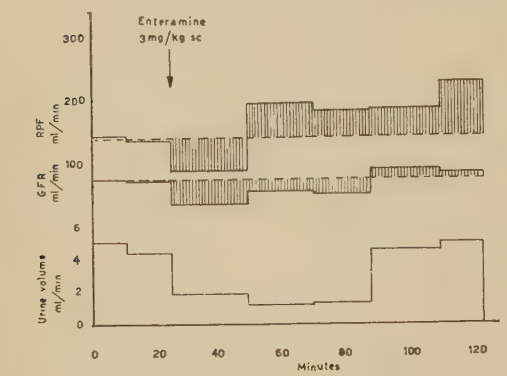

FIG. 3. Effect of 3 mg/kg of enteramine on urine volume, GFR and RPF of the hydrated dog.

urine flow under 1-0, 8 ml per minute. At constant initial urine flows, the higher doses of enteramine generally cause a stronger decrease of urine volume, but the antidiuretic effect is not directly correlated with the dose employed. The antidiuretic effect begins within 10 minutes after the subcutaneous or intravenous injection and lasts 40-60 min; successively the antidiuretic effect stops and the urine volume increases to, or even more, the preinjection values. Intavenously continuous infusion of enteramine (2-3 mg/kilo/hour) results in a more strong antidiuretic action.

Glomerular filtration rate shows a different behaviour according to the doses; in fact while the lower doses of enteramine (0, 1-0, 3 mg per kilo) do not significantly affect the filtration rate (Fig. 1, 2), the higher doses (0, 5-1-3 mg per kilo) are likely to reduce the GFR, (Fig. 3), the decrease being proportional to the dose employed. In this last case the decreased filtration rate, however, is not responsible by itself for the antidiuretic activity, inasmuch as even now the thiosulfate ratio of urine to plasma concentration (U thios./P thios.) increases markedly and more significantly than the change in GFR and the water to thiosulfate clearance (V/thios. Cl) constantly decreases, clearly indicating that enteramine determines an increase in the percentage of filtered water reabsorbed.

Renal plasma flow generally increases. The increase begins 20-30 minutes after the administration of enteramine and reaches its maximal rate during the diuretic phase which follows the reduction of the urine volume. It is manifest both in dogs infused with saline and distilled water and is present for doses as high as 1 mg per kilo even in those periods characterized by a slight decrease of GFR (Fig. 2). At higher doses the strong reduction of GFR meets with a concomitant decrease of PAH clearance; even in these high doses the diuretic phase is accompanied by a sharp increase of RPF (Fig. 3). The *filtration fraction* consequently decreases, this effect being obviously more evident in those periods when the filtration rate remains immodified and the plasma flow increases.

Discussion. The experiments here reported clearly emphasize the antidiuretic effect of enteramine, claimed by Erspamer *et al.*(3,14). The decrease of the urine flow in hydrated dogs is definite and the doses necessary to this effect are very low (in the order of 0, 1-0, 3 mg per kilo), certainly lower than those affecting the systemic blood pressure or those stimulating the smooth muscle. As regards the causal mechanism of the antidiuretic activity of enteramine, 3 main possibilities must be taken into account: a) the spasmogenic effect on smooth muscle of the bladder and the ureters b) the constriction of the afferent glomerular arteriole with reduction of GFR; c) the primitive increase of tubular reabsorption of water.

It has been demonstrated by Erspamer(12) and confirmed by subsequent trials, that enteramine is a spasmogenic substance and that the urinary excretory territory may become constricted under its action. What is to be stressed, however, is: 1) that the anti-

diuretic effect is already obtainable with doses which by themselves are not spasmogenic; 2) that it is generally assumed that the increased ureteral pressure meets with a decrease of GFR and a total reduction of the renal vascular bed(13). This second alternative will be discussed later.

The reduction of GFR which enteramine provokes in the rat led Erspamer to refer the antidiuretic effect of the substance to a constriction of the afferent bed of the glomerule. It is a matter of fact that the higher doses of enteramine cause in the dog a reduction of the filtration rate with consensual, though less evident, reduction of RPF, but these hemodynamic changes alone cannot explain the antidiuretic effect, insofar as the percentage of the filtered water reabsorbed is constantly increased by enteramine. Moreover enteramine may reduce urine volume without decreasing the GFR. and this effect occurs for the lower physiological doses.

The RPF consistently increased in the majority of the cases, only the higher doses of enteramine tending to determine a slight initial decrease of PAH clearance. In our opinion this fact leads again to cast some doubt on the prevailing role of the decrease of GFR in the mechanism of the antidiuretic effect of enteramine. Consequently, the antidiuretic effect of enteramine is to be referred to an increase of tubular reabsorption of water. We are now investigating the possible role of activation of the posterior pituitary, the relations between enteramine and the other antidiuretic substances in the blood, and the possible interference of Enteramine on water metabolism of the man in physiological and pathological conditions.

Summary. 1. Enteramine (5 hydroxytryptamine) decreases the urine volume of hydrated dogs at doses which do not stimulate the smooth muscle and do not modify the systemic blood pressure. The lower doses (0, 1-0, 3 mg per kilo) subcutaneously or intravenously decrease the urine volume without affecting the GFR, while higher doses reduce also the filtration rate; both lower and higher doses increase the RPF. 2. The above described experiments suggest that the antidiuretic effect of enteramine in the dog derives principally from an increased reabsorption of water from the renal tubule.

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Preservation of Monkey Testicular Tissue with Human Albumin.* (20197)

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During the course of studies designed to obtain multiplication of human fibroblasts in roller tubes, human and bovine albumin were substituted for horse serum in the culture medium. As a result of these experiments three observations were made. 1) Outgrowth of fibroblasts occurred in a medium consisting of 1.25% human albumin, Simms-Hanks' solution, and chick-embryo extract. 2) Tissues grown in media containing varying quantities of unheated horse serum became granular and showed evidence of deterioration in less than two weeks. These disintegrative changes were prevented by adding albumin. 3) When 10% albumin was employed, the original tissue in the roller tube did not proliferate but appeared unusually well preserved after incubation at 37°C for one month. These facts stimulated interest in the use of albumin for maintaining tissue in a viable form and as a constituent of culture media.

Method. The method of roller tube culture was essentially the same as that already reported(1). Commercial lots of 25% salt-poor human albumin in buffered diluent were kindly furnished by Squibb and by Cutter Laboratories. Albumin preserved with merthiolate is toxic for fibroblasts. Chick-embryo extract was prepared after the method recommended by Youngner *et al.*(2).

Experimental. A single testicle from an immature rhesus monkey was minced into pieces suitable for roller tube cultures. Portions of the tissue were embedded and incubated the same day. The remainder was placed in the refrigerator in 50 ml of Hanks' salt solution which contained 2.5% human albumin, and streptomycin and penicillin in concentrations of 0.1 mg and 100 units respectively. Six weeks later pieces of the tissue were removed, washed and embedded in chick plasma clots. The culture medium consisted of 1.25% human albumin, 10% chick-embryo extract, and 10% unheated horse serum, in Simms-Hanks' mixture,† and the usual concentration of penicillin and streptomycin. The same process was repeated after the fragments had remained in the refrigerator for 7 and 8 weeks. The cultures were examined daily and the experiment was terminated when the tissue explants showed a well marked zone of fibroblastic proliferation measuring 1-2 mm. Initial growth was observed within 2 days in some fragments; by 4 days proliferation was visible grossly, and in one week or less the zone of cell outgrowth had reached 1.5 to 2 mm. Good outgrowth of fibroblasts was obtained from practically all fragments despite the fact that the minced testicle had been held in the refrigerator for as long as 8 weeks.

In a second experiment monkey testicular tissue was minced and divided into 2 portions. One was refrigerated in 25 ml of Simms-Hanks' solution and the other was placed into an equal amount of Hanks' balanced salt solution containing 2.5% human albumin. The results of tests for viability, after storing testicular tissue in the refrigerator for a total of 5 weeks, are shown in Table I. The specimens kept in albumin for 5 weeks began to grow in 3 days and reached 1-2 mm by 6 days. The fragments held in Simms-Hanks' solution remained viable for only two weeks.

Human albumin was also incorporated in culture media and the results are presented in Table II. Freshly obtained monkey testicle was grown in varying quantities of 50% chick-embryo extract and human albumin, and made up to proper concentration with Simms-Hanks' solution. Rapid and adequate multiplication of monkey fibroblasts took place over a wide range of albumin or chick-embryo extract concentrations in the absence of horse serum. A minimum of 10% chick-embryo was required and a concentration of 1.25%

* Aided by a grant from the National Foundation for Infantile Paralysis.

† 1 part Simms' serum ultrafiltrate and 3 parts Hanks' balanced salt solution.

TABLE I. Preservation of Monkey Testicular Tissue in 2.5% Human Albumin.

Refrigeration, wk	Growth after 10 days at 37°C: Simms-Hanks' solution, Tubes 1	2	3	4	5	6	2.5% human albumin, Tubes 1	2	3	4	5	6
1	4/8	3/6	3/6				8/8	8/8	8/8			
2	0/6	1/8	2/6	1/6	2/6	5/8	6/6	6/6	7/7	6/6	8/8	7/7
3	0/6	0/5	0/5	0/7			4/4	8/9	6/6	8/8		
5	0/6	0/8	0/7	0/8	0/6	0/7	5/5	4/4	6/6	8/9	5/6	5/5

Denominator = total fragments; numerator = No. growing.

TABLE II. Effects of Varying Quantities of Human Albumin and Chick-Embryo Extract on Growth of Monkey Fibroblasts.

Medium	% albumin	% CEE	Fragments growing, Tubes 1	2	3	Degree of growth	Initial & final pH
1			9*	9	10	+	7.5
	1.25	25	10	10	10	4+	6.9
2			10	10	10	+	7.6
	1.25	10	10	10	10	4+	7.0
3			10	10	8	+	7.6
	1.25	5	10	10	10	4+	7.0
4			4	8	8	±	7.6
	1.25	1	10	10	10	2+D	7.2
5			0	4	3	±	7.6
	1.25	0	0	10	9	+D	7.2
6			0	0	0	—	6.8
	7.5	10	9	10	10	+	7.0
7			4	6	6	±	7.0
	5.0	10	10	10	10	3+	7.0
8			9	9	10	+	7.3
	2.5	10	10	10	10	4+	7.1
9			10	10	9	+	7.5
	1.25	10	10	10	10	4+	7.1
10			9	9	8	±	7.9
	.25	10	10	10	10	4+	7.0
11			6	10	9	±	8.0
	0	10	10	10	10	2+D	7.1
12			4	6	8	+	7.7
	1.25	10†	10	10	10	4+	7.0
13			10	9	10	+	8.0
	0	10†	10	10	10	3+D	7.0
14			8	6	4	±	8.1
	0	0	10	10	10	2+D	7.2

CEE = 50% chick-embryo extract.
* No. indicates growth of fibroblasts from total of 10 explants.
† 10% unheated horse serum added.
± Early proliferation of single fibroblasts.
+ = Definite outgrowth from numerous margins of the explants.
2+ = ½ mm zone of fibroblasts; 4+ = 1-2 mm zone.
First reading after 2 days incubation at 37°C; second reading after 8 days. Final pH obtained after 8 days incubation at 37°C.
D = Disintegrative signs as rounding of cells, granularity.

albumin was sufficient for good proliferation of cells. The pH was not particularly critical unless extremes were reached (media 6, 11, and 14). In a duplicate experiment, made at the same time, the pH of media 6, 7, 8 and 9 was adjusted with sodium carbonate to 7.4 and growth of fibroblasts was obtained in the presence of 7.5% albumin, but not to the same degree as with the lesser amounts. Changes in pH during growth did not appear

to influence the development of disintegrative changes.

Discussion. The data presented are of interest from the point of view of preservation of monkey testicular tissue in the refrigerator for prolonged periods of time (5 to 8 weeks). This fact should prove helpful in those laboratories where such tissues are used for the isolation and identification of poliomyelitis viruses. The mechanism of action of albumin as a protector of fibroblasts has not been investigated. An analogous situation is the stimulating effects of either whole serum or the albumin fraction on growing tubercle bacilli as originally observed by Youmans and confirmed by others(3-6). In this instance the albumin was thought to neutralize toxic fatty acids and other substances(3,4). To the authors' knowledge human albumin has not been previously used as a constituent of media for the cultivation of cells. Sanford, *et al,*(7) have recently reported an unsuccessful attempt to fortify a medium containing chick-embryo extract and horse serum ultrafiltrate with 6% crystalline bovine albumin. This mixture was not better than whole horse serum for the quantitative cultivation of the L strain of mouse cells. We have utilized mixtures of bovine albumin and horse serum for growing human fibroblasts. Proliferation was equal to that seen with human albumin but degenerative changes appeared earlier.

Summary. Minced immature rhesus testicular tissue remained viable for a period of 8 weeks when refrigerated at 5°C in Hanks' basic salt solution containing 2.5% salt-poor human albumin. Some observations on the use of human albumin as a constituent of tissue culture media have been recorded.

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Thyroid and Vitamin B_{12} Interactions in the Mouse.* (20198)

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Low doses of thyroid-active substances have been shown to significantly increase the rate of growth in young mice for limited periods of time(1-3). Daily injections of .01-.03 mg of crystalline thyroxine sodium or feeding .04-.32% of iodinated casein in the ration for a 5-week period increased body weight gains by about 28% over untreated control mice(2). This was accompanied by an increase in tissue protein and water, a decrease in fat and greater body length. Excessive doses of thyroid-active materials however, inhibit body growth in young mice, an effect which can largely be counteracted by supplementing the diet with "animal protein factor" or vit. B_{12} (4,5). It was of interest therefore, to determine in mice the interactions betwen a dose of a thyroid-active substance which would increase growth rate and vit. B_{12}.

Methods. A total of 36 immature, male Rockland mice and 45 immature, male Car-

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worth mice were used in 2 separate experiments. The animals were divided into uniform groups by weight and were fed the following vit. B_{12}-deficient ration: yellow corn meal, 35%; ground wheat, 25%; linseed oil meal, 10%; soybean oil meal,§ 20%; alfalfa leaf meal, 6%; brewers' yeast, 3%; and table salt, 1%. In the first experiment, 4 groups of Rockland mice were treated as follows: controls, untreated; 100 μg vit. B_{12}‖ per kilo of ration; .025% Protomone;¶ 100 μg vit. B_{12} and .025% Protomone. In the second experiment, 5 groups of Carworth mice were similarly treated except that two levels of vit. B_{12} were fed together with the Protomone. Body weights were recorded every 2 days in all the mice and food consumption was measured only in the mice of the first experiment. All mice were housed in metal cages with raised screen bottoms in an air conditioned room maintained at 75 $\pm$ 2°F.

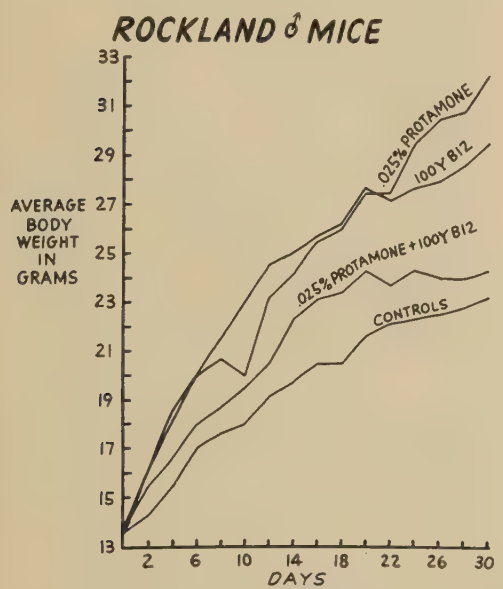

FIG. 1. Growth curves of Rockland mice receiving vit. B_{12} and/or Protamone.

§ Low fiber, solvent extracted, containing 50% protein. Manufactured by Archer-Daniels-Midland Co., Minneapolis, Minn.

‖ Vit. B_{12} was made available through the courtesy of Dr. L. Michaud of Merck and Co., Rahway, N. J.

¶ Iodinated casein product containing thyroxine. Kindly furnished by Cerophyl Labs., Kansas City, Mo.

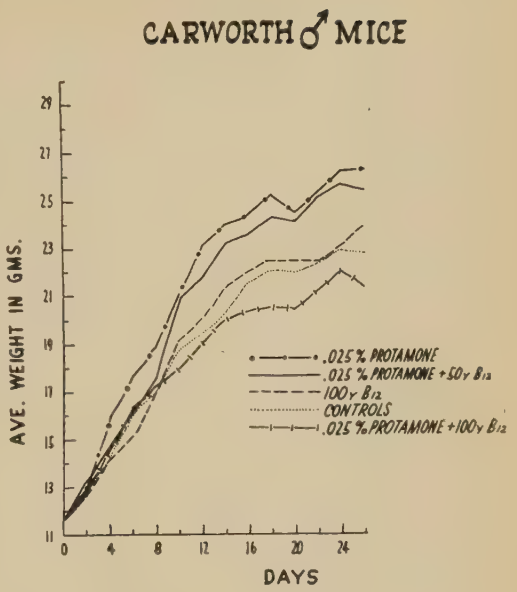

FIG. 2. Growth curves of Carworth mice receiving vit. B_{12} and/or Protamone.

Results. The growth curves of the mice in the first experiment are shown in Fig. 1. The average body weight per mouse at the beginning of the experiment was 13.6 $\pm$ 0.5 g. The average body weight and total food consumption per mouse at the end of 30 days were as follows for each group: controls, 23.2 $\pm$ 0.8 g and 96.0 g; .025% Protamone, 32.3 $\pm$ 1.4 g and 161.7 g; 100 μg vit. B_{12}, 29.5 $\pm$ 1.5 g and 120.3 g; .025% Protamone and 100 μg vit. B_{12}, 24.3 $\pm$ 1.8 g and 121.2 g. The average food intake in grams per gram of body weight gain for each of the 4 groups was as follows: 10.0, 8.7, 7.5 and 11.5. Thus the efficiency of converting food into body weight gains was greatest for the groups receiving either Protamone or vit. B_{12} and least for the group receiving both substances.

The growth curves of the mice in the second experiment are shown in Fig. 2. These mice weighed 11.7 $\pm$ 0.4 g each at the beginning of the experiment. The average final body weight per mouse for each group was as follows: controls, 22.6 $\pm$ 0.6 g; 100 μg vit. B_{12}, 24.0 $\pm$ 0.8 g; .025% Protamone, 26.3 $\pm$ 0.8 g; .025% Protamone and 50 μg vit B_{12}, 25.7 $\pm$ 0.6 g; .025% Protamone and 100 μg. vit B_{12}, 21.6 $\pm$ 1.0 g. It can be seen that even though 100 μg of vit. B_{12} did not signi-

ficantly increase the growth rate of these mice, this amount of vit. B_{12} fed together with Protamone completely eliminated the favorable action of the latter on growth.

Discussion. These data are in agreement with previous reports that a mild degree of hyperthyroidism increases the growth rate of young mice(1-3). A maximum difference in average body weight of about 2.5 g between thyroid-treated and untreated mice was noted in these earlier studies, whereas differences of 8.9 g and 3.8 g were seen in the present 2 experiments. An analysis of the rations used by the previous investigators showed that they contained considerable quantities of "animal protein" and presumably of vit. B_{12}, whereas the basal ration used in the present study was deficient in vit. B_{12}. It seems probable therefore, that the differences in the growth response of these mice to thyroid-active substances were influenced by the levels of vit. B_{12} in the diets. Swine may represent another species in which a growth response to a mild degree of hyperthyroidism is enhanced if the ration is deficient in vit. B_{12}. Beeson(6) reported that a small dose of Protamone was very effective in stimulating increased growth in pigs when the ration was deficient in vit. B_{12}, but that the same dose of Protamone was completely ineffective in stimulating growth in the presence of adequate vit. B_{12}.

No satisfactory explanation can be given at present for the apparent antagonism observed between vit. B_{12} and Protamone in these mice. It has been suggested that a limiting factor or factors in the diet, *i.e.* methionine, may account for this phenomenon (Dr. A. White, personal conversation), but further research will be necessary to resolve this question.

Summary. 1. The effects of a mild degree of induced-hyperthyroidism or vit. B_{12} or both on growth rate were determined in 81 immature male mice fed a vit. B_{12}-deficient ration, in two experimental trials of 26 or 30 days duration. 2. Feeding .025% Protamone (iodinated casein) or 100 μg of vit. B_{12}/kilo of ration each increased the growth rate of the mice, and Protamone was more effective than vit. B_{12} in this respect. Each substance increased food intake and enhanced the efficiency of converting food into body weight gains. When Protamone and vit. B_{12} were fed together however, the favorable effects of either on growth were largely eliminated and efficiency of food utilization was reduced. No satisfactory explanation can be offered for this phenomenon at this time.

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Effect of Hormone Therapy on Body Weight During Protein Depletion and Repletion.* (20199)

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There is evidence that androgens(1) insulin (2,3), and growth hormone under certain conditions(4-7) promote protein anabolism whereas adrenocortical steroids of the cortisone type augment nitrogen excretion(8,9). It was felt that these hormones (if involved in the regulation of protein metabolism) might alter the rate of loss or gain in body weight during the course of protein depletion and repletion. In earlier experiments with rats, neither the weight loss during depletion nor the gain in body weight during repletion was influenced by testosterone treatment(10). In the present communication data are presented on the effects of insulin, growth hormone, and cortisone administration on the change in body weight of rats under conditions similar to the above.

Procedure. Two experimental rations were employed in the present investigation: diet A and diet B. Diet A was a protein-free ration of the following composition: sucrose, 80%; salt mixture,‡ 5%; cellulose,§ 5%; cottonseed oil (Wesson), 8%; and wheat germ oil (Vio-Bin), 2%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 100 mg; biotin, 5 mg; 2-methyl-naphthoquinone, 5 mg; folic acid, 10 mg; para-amino-benzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; and choline chloride, 2 g. Diet B was similar to diet A except that casein‖ was incorporated in this ration at a level of 24% of the diet, replacing an equal amount of sucrose. Each rat also received once weekly 4.5 mg alpha tocopherol acetate and a vitamin A-D concentrate¶ containing 150 U.S.P. units of vit. A and 15 U.S.P. units of vit. D. Eighty-four male rats of the Wistar strain which had been raised from weanling on a natural food ration** were selected at an average body weight of 252.7 g (range 201-290 g) for the present experiment. Animals were placed in individual metal cages with raised screen bottoms and were fed for 15 days the protein-free ration diet A. For the next 15 days each rat received the complete ration diet B. Animals were fed *ad libitum* and food consumption was determined for each rat. Rats were divided into 7 groups of 12 animals:

Group I was administered 0.5 cc saline solution daily during both the depletion and repletion period. *Group II* received saline solution during the depletion period and 500 μg of growth hormone†† daily during the reple-

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† With the School of Medicine, University of S. California, Los Angeles, and Van Camp Sea Food Co., Terminal Island, Calif.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

§ Ruffex, Fisher Scientific Co., St. Louis, Mo.

‖ Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

¶ Nopco Fish Oil Concentrate, assaying 800000 U.S.P. Units of vitamin A and 80000 U.S.P. units of vit. D/g.

** Purina Chow.

†† Somatrofin (STH-Horner), Frank W. Horner, Montreal, Canada. When assayed in hypophysectomized female rats, 10 μg of this material daily, for 10 days resulted (according to Dr. L. Mitchell of Frank W. Horner) in average increment of 11 g body weight. This material was dissolved in alkaline aqueous solution and the volume adjusted to contain 1 mg Somatrofin/cc.

TABLE I. Comparative Effects of Saline, Cortisone, Insulin, and Growth Hormone Administration on the Loss in Body Weight of Adult Rats During Protein Depletion. (15 days on protein-free diet.)

Series	No. of animals	Initial body wt, g	Avg loss in body wt,* g	Avg food consumption/rat during depletion, g
Saline Groups I, II, III, IV	48	252.3	56.1 ± 1.2 (47)	184
Cortisone Group V	12	253.5	53.2 ± 2.8 (12)	186
Insulin Group VI	12	253.9	55.4 ± 1.7 (11)	181
Growth hormone Group VII	12	252.3	58.3 ± 2.0 (12)	179

* Including stand. dev. of the mean.
Values in parentheses indicate No. of animals which survived and on which averages are based.

TABLE II. Summary of Data—Protein Repletion Experiment. (15 days on protein repletion diet.)

No. of animals	Material inj. during depletion period	Material inj. during repletion period	Avg food consumption/rat during repletion period, g	Avg gain in body wt over depletion wt,* g	Avg gain/100 g food ingested,* g
12	Saline	Saline	304.4	100.3 ± 2.9 (12)	33.0 ± 0.7
12	"	Growth hormone	325.5	117.4 ± 4.0 (12)	36.0 ± 0.8
11	"	Insulin	319.0	101.4 ± 6.6 (11)	31.7 ± 1.0
12	"	Cortisone	320.4	104.0 ± 4.9 (11)	32.5 ± 0.9
12	Cortisone	Saline	317.6	96.5 ± 7.1 (12)	30.3 ± 1.2
11	Insulin	"	316.7	100.8 ± 7.0 (8)	31.8 ± 1.3
12	Growth hormone	"	318.4	102.8 ± 5.1 (11)	32.3 ± 1.1

* Including stand. dev. of the mean.
Values in parentheses indicate No. of animals which survived and on which averages are based.

tion period. *Group III* received saline solution during the depletion period and 0.6 units insulin‡‡ daily during the repletion period. *Group IV* received saline solution during the depletion period and 2.5 mg cortisone acetate§§ daily during the repletion period. *Group V* received cortisone acetate in the above dosage during the depletion period and saline solution during repletion. *Group VI* received insulin in the above dosage during depletion and saline solution during repletion. *Group VII* received growth hormone in the above dosage during depletion and saline solution during the repletion period. The saline and hormone solutions were administered in divided doses twice daily. All injections were made subcutaneously.

Results are summarized in Tables I and II. Findings indicate that during the depletion period the rate of loss as well as the average total loss in body weight was similar for all

‡‡Protamin Zinc Insulin (Lilly) 0.6 unit diluted to 0.5 ml with water.

§§ Saline solution of cortone acetate, Merck and Co., Rahway, N. J., containing 25 mg cortisone acetate/cc. This material was diluted with saline solution to a concentration of 5 mg cortisone acetate/cc.

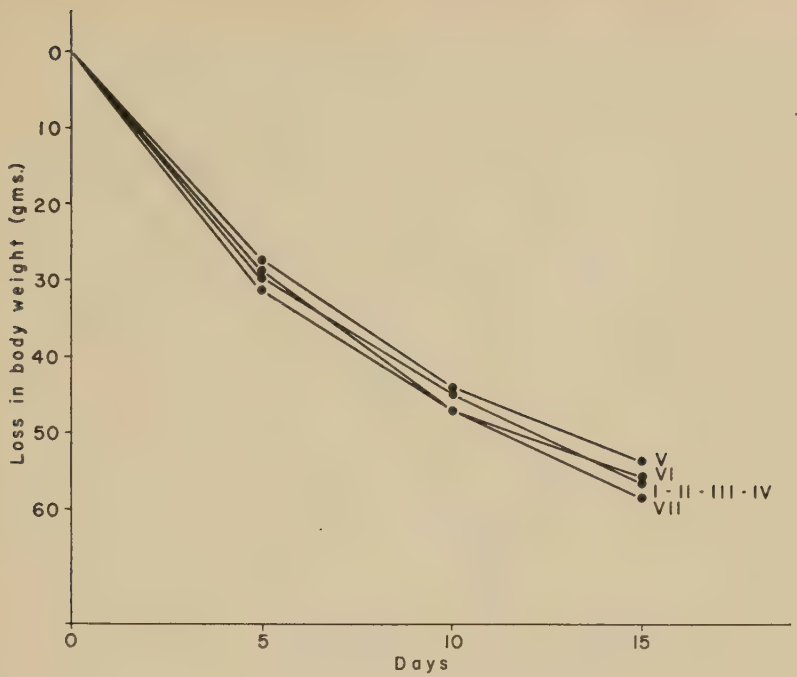

FIG. 1. Depletion experiment.
Group I, II, III and IV on saline; Group V, cortisone treated; Group VI, insulin treated; Group VII, growth hormone treated.

groups (Table I; Fig. 1). In the repletion period the initial body weight was regained in all groups after 7 days of feeding. During this period of repletion the growth increment was practically identical for all groups (Fig. 2). Subsequent to this period, however, the growth increment of animals administered growth hormone during the repletion period was greater than that of other groups. Average food consumption per rat was similar in all groups in both the depletion and repletion period. The efficiency of food utilization as judged by gain in weight per 100 g of food ingested during repletion was slightly greater for animals in group II than for other groups tested.

Discussion. Previous findings indicate that the change in body weight of rats during protein depletion and repletion was not affected by testosterone administration(10). Present data indicate that the administration of insulin and cortisone acetate was similarly ineffective. Growth hormone was without effect on the loss in body weight during protein depletion and on the gain in body weight during repletion until such time as initial body weight was regained. Once pre-depletion weight was attained, however, the growth increment was greatest in the group administered growth hormone. These findings suggest that under conditions of the present experiment exogenous sources of testosterone, insulin, cortisone, or growth hormone were without significant effect on protein depletion and repletion in the rat (as judged by change in body weight), at least in the dosage at which these hormones were administered. It is possible that larger or smaller doses of the various hormones may have yielded different results. The dosages employed, however, appeared to be of physiological "magnitude" and represented quantities which when injected into rats in other experiments showed typical hormonal effects. It is also possible that the above hormones (although without effect on body weight during protein depletion and repletion) may have exerted differential effects on the composition of the tissues. Kochakian's observation(11) that testosterone propionate hastened the replenishment of protein

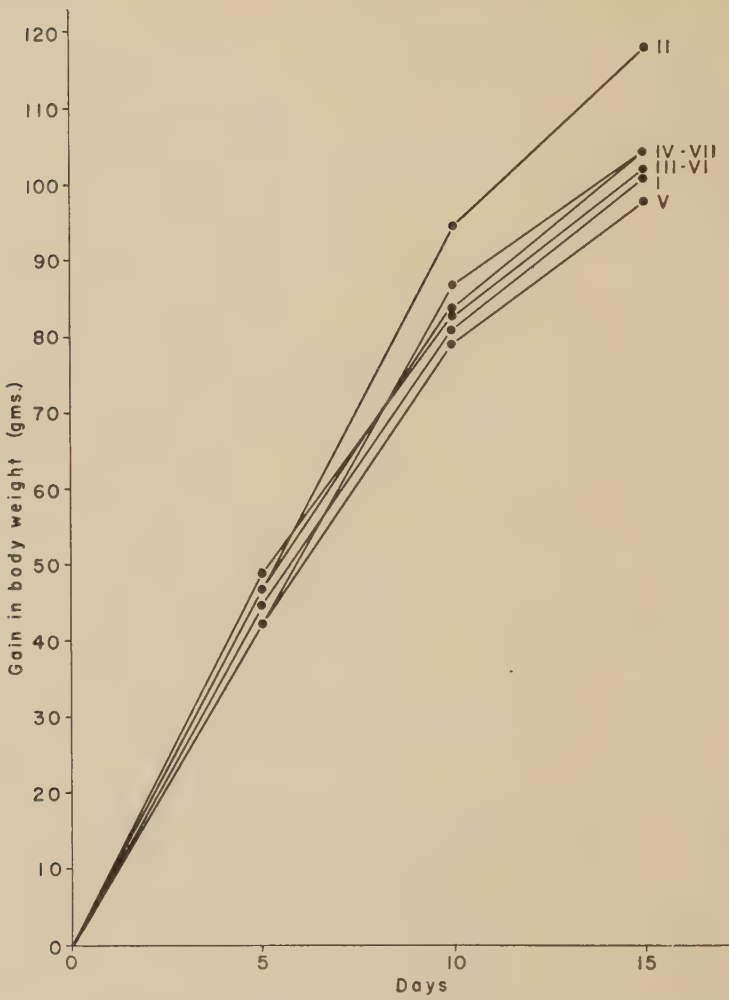

FIG. 2. Repletion experiment.
Group I, saline treated (during depletion, saline); Group II, growth hormone treated (during depletion, saline); Group III, insulin treated (during depletion, saline); Group IV, cortisone treated (during depletion, saline); Group V, saline treated (during depletion, cortisone); Group VI, saline (during depletion, insulin); Group VII, saline (during depletion, growth hormone).

in starved rats may be pertinent in this regard.

It seems likely that during protein depletion and repletion all available regulative mechanisms including hormonal factors are mobilized by the body in an attempt to maintain or restore homeostasis. This increased anabolic tendency finds its expression in the fact that the body weight lost during 15 days of depletion is restored within 5 to 7 days during repletion. It would seem plausible that in the normal intact animal the anabolic mechanisms of the tissues have an upper limit which cannot further be increased by exogenous hormone supplies.

Summary. The effects of growth hormone, insulin, and cortisone acetate administration was determined on the change in body weight of rats during protein depletion and repletion. Neither the weight loss during depletion nor the gain in body weight during repletion was affected by the administration of these hormones. After pre-depletion weight was regained, however, the growth increment for animals administered growth hormone was

larger than for the other groups.

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Complex Formation and Chemical Specificity of Boric Acid in Production of Chicken Embryo Malformations.* (20200)

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Boric acid, when injected into the yolk sac of developing chicken eggs, acts as a potent teratogenic substance(1). Following treatment at 4 days of incubation a typical syndrome arises, with features of shortening (often extreme) of the mandible, facial coloboma and cleft palate, shortening and bending of the tarsometatarsus, and poor development of the toes or their complete lack, the fourth toe being affected preferentially. Length of femur and tibiotarsus may also be reduced, depending on the amount of injected boric acid(2). Among hatched chicks the most prominent symptom is curled-toe paralysis.

These effects of boric acid on development assume particular interest on account of varied evidence, also previously discussed, pointing to the conclusion that the malformations are caused by an interference with the embryos' supply of riboflavin. In the experiments to be reported here we sought information on two points: 1. Will substances which are known to form complexes with boric acid, reduce or abolish the teratogenic qualities of the latter when they are dissolved in the boric acid solution prior to treatment or when they are used as supplements? 2. Is the teratogenic action specific for boric acid or is it, more generally, a quality of boron?

Methods. Sterile solutions of the compounds to be tested were made up in saline with 0.25% phenol added. The injections were given into the yolk sac in volume of 0.05 cc using a tuberculin syringe and No. 27 needle. The experiments were carried through to hatching. The results are based on all survivors of the 13th day of incubation.

Results. The results of experiments with compounds which are known to form complexes with boric acid are shown in Tables I and II. In a first series of tests (Table I) the teratogenic effects of boric acid were compared with the consequences of simultaneous but separate injection of boric acid and either D-ribose or pyridoxine hydrochloride. It can be seen that the addition of D-ribose or pyridoxine hydrochloride increased the chances of embryos to develop normally and reduced the incidence of every one of the different types of malformations. Considered individually, many of the differences between the group treated with boric acid and the three other groups are on the borderline of significance; but, if all features are taken into consideration, the differences become highly significant. D-

* Supported by a grant from the American Cancer Society, on recommendation by the Committee on Growth, National Research Council.

TABLE I. Experiments with 2.5 mg/egg Boric Acid Alone and Supplemented by Simultaneous Injection of 2.5 or 10 mg D-ribose or 5 mg Pyridoxine Hydrochloride.

	Boric acid	Boric acid and 2.5 mg D-ribose	Boric acid and 10 mg D-ribose	Boric acid and 5 mg pyridoxine hydrochloride
Survivors of 13th day	60	73	72	112
Normal, %	36.7 ± 6.2*	49.3 ± 6.0	54.2 ± 6.0	50.9 ± 4.8
Simple beak defects, %	15.0 ± 4.6	9.6 ± 3.5	11.1 ± 3.8	4.5 ± 2.0
Complex beak defects, %	43.3 ± 6.4	38.3 ± 5.8	26.3 ± 5.1	27.7 ± 4.3
Tibiotarsus bent, %	6.7 ± 3.2	2.7 ± 1.9	1.4 ± 1.4	0
Abnormal tarsometatarsus, %	11.6 ± 4.1	4.1 ± 2.4	2.8 ± 2.0	2.7 ± 1.6
Abnormal toes, %	20.0 ± 5.2	9.6 ± 3.5	7.0 ± 3.0	3.6 ± 1.8

* Standard errors.

TABLE II. Results of Injecting Solutions Containing 2.5 mg Boric Acid Alone or with Addition of 2.5, 10 or 20 mg D-sorbitol Hydrate.

	Boric acid alone	Boric acid containing 2.5 mg D-sorbitol hydrate	Boric acid containing 10 mg D-sorbitol hydrate	Boric acid containing 20 mg D-sorbitol hydrate
No. treated	78	93	90	243
Hatched, %	19.2 ± 4.4*	38.7 ± 5.1	61.1 ± 5.1	54.3 ± 3.2
Curled-toe paralysis (among hatched), %	33.3 ± 5.3	19.4 ± 4.1	7.3 ± 2.7	0
Survivors of 13th day	60	86	81	194
Normal, %	36.7 ± 6.2	40.7 ± 5.2	79.0 ± 4.6	99.0 ± .7
Simple beak defects, %	15.0 ± 4.6	7.0 ± 2.7	8.6 ± 3.1	.5 ± .2
Complex beak defects, %	43.3 ± 6.4	47.7 ± 5.3	8.6 ± 3.1	0
Tibiotarsus bent, %	6.7 ± 3.2	2.3 ± 1.6	0	0
Abnormal tarsometatarsus, %	11.6 ± 4.1	12.8 ± 3.5	1.2 ± 1.2	0
Abnormal toes, %	20.0 ± 5.2	9.3 ± 3.1	2.4 ± 1.7	0
Syndactylism, %	1.7 ± 1.7	4.7 ± 2.2	0	0

* Standard errors.

ribose was more effective in preventing defects at the higher than at the lower dosage level.

We obtained still clearer results when, prior to injection, D-sorbitol hydrate was dissolved in the boric acid solution (Table II). At the 2.5 mg level the effect of D-sorbitol was slight and its significance uncertain, except that survival and hatching were definitely improved; at the 10 mg level the incidence of all abnormalities was much decreased and the percentage of normal chicks correspondingly raised; at the 20 mg level the teratogenic consequences of boric-acid treatment had been very nearly abolished. A comparison of the data in Tables I and II shows that at the same dosage levels D-sorbitol was more effective than D-ribose in reducing the teratogenic action of boric acid. This may have been due to the different manner of administration, but it is also known(3) that D-sorbitol is more reactive with boric acid than D-ribose.

In earlier experiments it had been observed that the teratogenic effects of boric acid can be mitigated by the addition of riboflavin. The present tests with other polyhydroxy compounds, known to undergo complex formation with boric acid, confirm the assumption that in the form of complexes boric acid ceases to be teratogenic. Carbohydrates which do not form complexes with boric acid, *e.g.* glucuronic acid lactone, are ineffective. Tests with D-sorbitol illustrate, on the other hand, that with increasing amounts of this acyclic polyol the teratogenic effectiveness of boric acid declined in a quantitative manner.

A study of the chemical specificity of boric acid is made difficult by the dearth of water-soluble boron compounds other than borates. All borates, on the other hand, with one exception to be dealt with presently, liberate boric acid in solution. Tests were made with sodium metaborate (2.5 and 5 mg/egg) and with sodium borate (3.5 mg/egg). At the lower dosage level sodium metaborate was responsible for a few cases of curled-toe paralysis, but did not produce morphological defects;

TABLE III. Experiments with Boric Acid, Triethanolamine Borate* and Sodium Aluminate. Injections at 96 hr incubation.

	Boric acid, 2.5 mg	Triethanolamine borate, 5 mg	Sodium aluminate 2.5 mg	Sodium aluminate 5 mg
Survivors, 13th day	83	132	100	81
Normal, %	1.2 ± 1.2†	34.1 ± 4.1	89.0 ± 3.1	82.7 ± 4.2
Simple beak defects, %	6.0 ± 2.6	12.9 ± 2.9	0	1.2 ± 1.2
Complex beak defects, %	67.5 ± 5.2	49.2 ± 4.4	0	0
Tibiotarsus bent, %	2.4 ± 1.7	0	4.0 ± 2.0	16.0 ± 4.1
Abnormal tarsometatarsus, %	33.7 ± 5.2	20.5 ± 3.5	4.0 ± 2.0	2.5 ± 1.7
Abnormal toes, %	18.1 ± 4.2	3.8 ± 1.7	0	0
Syndactylism, %	4.8 ± 2.3	.8 ± .8	0	0

* Prepared by Sigma Chemical Co., St. Louis. † Standard errors.

at the 5 mg/egg level typical malformations occurred, but with low incidence. Experiments with 3.5 mg/egg sodium borate (about equivalent in boron to 2.5 mg/egg boric acid) yielded, on the other hand, results that were quantitatively and qualitatively very similar to those obtained with the injection of 2.5 mg/egg boric acid. For the reason indicated, it is difficult to know how much of the teratogenic action of these borates was due to boric acid.

Brown and Fletcher(4) have reported the synthesis of triethanolamine borate which is hydrolyzed only very slowly in dilute acid and little, if at all, in neutral water. At the stage of our experimentation the yolk pH is about 6. We have, therefore, made a test comparing the effects of boric acid (2.5 mg/egg) with those of triethanolamine borate (5 mg/egg). The boron content of the borate was somewhat lower than that of the boric acid; higher doses proved to be too toxic. Compared with boric-acid treatment, injection of triethanolamine borate allowed more embryos to complete development normally and the various kinds of malformations occurred with lower frequency (Table III). Yet, the incidence of abnormalities was high and the defects were morphologically identical with those produced by boric acid. Hence, this boron compound shares the teratogenic qualities of boric acid.

It seemed of interest to determine if aluminum, next to boron in the periodic system, interferes with the development of the chicken embryo in a fashion similar to that of boron. The results of experiments with sodium aluminate are reproduced in Table III. The teratogenicity of the aluminum salt was, on the whole, low; but there were some instances of shortened and bent tarsometatarsus, very similar to the characteristic effects of boron compounds. Syndactylism, reduction of toes and complex beak defects were not encountered. Bending of the tibiotarsus, on the other hand, occurred with surprising frequency (χ^2 of difference in incidence after 2.5 mg boric acid and 5 mg sodium aluminate was 7.974, with $P < .01$). Among the hatched chicks we found a few cases of typical curled-toe paralysis. There is little doubt that the action of the aluminum salt is related to, if much weaker than, that of the boron compounds. Our data suggest that in the appendicular skeleton sodium aluminate tended to interfere more proximally with development than do boric acid and other boron compounds.

Summary. Polyhydroxy compounds (D-ribose, pyridoxine hydrochloride, D-sorbitol hydrate), known to form complexes with boric acid, were tested for their effect on the teratogenic action of boric acid during development of the chicken embryo. It was found that complex formation reduces or abolishes the teratogenic qualities of boric acid. Triethanoalmine borate produced the same malformations as did boric acid. Injected sodium aluminate caused few developmental defects, but they seem to be related to those brought about by boron compounds. Our results support the working hypothesis that boric acid interferes with normal development by complex formation in ovo with polyhydroxy compounds, thereby producing symptoms resembling riboflavin deficiency. Additional evidence suggesting that in our material boric acid acted

on coenzymes rather than on enzymes was presented earlier.

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Hepatic Circulation in Hemorrhagic Shock in the Rat.* (20201)

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Intrahepatic vasoconstriction during hemorrhagic shock has been demonstrated in the dog by measurements of portal-caval pressure gradients(1,2) and by x-ray visualization of the intrahepatic veins delineated by thorotrast injection(2). This communication deals with experimental hemorrhagic shock in rats in which the reactions of the sinusoids and the pre-sinusoidal and post-sinusoidal venules were observed by direct microscopy of the transilluminated liver during a prolonged period of hypotension and after transfusion. Data are included on portal venous pressures.

Methods. Pressure in the portal venous bed was measured by a saline manometer connected to a cannula in a mesanteric vein. The ligature on the cannula also secured the accompanying artery to prevent blood loss into the area of obstructed venous return. A segment of intestine about an inch long was thus deprived of blood flow, but the remainder of the intestinal circulation was not disturbed. The incision was closed about the catheter and the rat was allowed to recover from ether anesthesia. After control readings were taken, irreversible hemorrhagic shock was produced as previously described(3). In a number of these rats, *thorotrast* was injected through the catheter to permit x-ray demonstration of the portal vein and its branches. For *direct microscopy,* the liver edge was exposed through a transverse subcostal incision, transilluminated with the aid of a curved quartz rod conducting light from a 200-watt tungsten filament lamp, and examined at a magnification of 150 x. The tip of the rod and the exposed liver were bathed in flowing gelatin-Ringers solution at 37°C. A metal bracket, curved to fit the dome of the liver and notched to avoid vena cava and aorta, was inserted between liver and diaphragm and clamped securely to minimize respiratory motion. Control observations showed that the presence of the bracket did not change vessel size or blood flow. Estimation of change in vessel size was aided by a micrometer scale in the ocular lens (each division representing 7.7 micra in the field). To make these observations, it was necessary to avoid initial heparinization. Hence these rats were bled in accordance with the criteria described(3) but they were not cannulated for continuous measurement of arterial pressure until ready for transfusion.

TABLE I. Portal Venous Pressures in Rats before and after Bleeding and Blood Replacement. 11 experiments.

Portal venous pressure (cm H_2O)			
Before bleeding	During hypotension	After blood replacement	After additional transfusions
17	8.5	15.5	—
15	9	—	—
10.5	7.5	10.4	—
12	7	9	11.5
15	10	16	26
16	10	16	16
17.5	10	18	17.5
14.5	10	15	18
17	11	15	17.2
14.5	9.5	15	22.0
15.1	9.0	15.2	15.9
Mean 15	9.0	14.5	—

Results. Portal venous pressure (Table I). Portal pressures fell from the mean control level of 15 cm H_2O(4) to a mean of 9 cm H_2O

* The investigation was aided in part by a research grant from the U. S. Public Health Service.

during hemorrhagic hypotension and returned to the prebleeding level after transfusion. Eight rats were given additional transfusions in volumes equal to 25% to 100% of their estimated initial blood volume; in only 2 did this procedure elevate the portal venous pressure above 18 cm H_2O.

X-ray visualization of the intrahepatic portal bed. The portal vessels were observed to constrict to 50-70% of the control cross-sectional area. Blood replacement after several hours of hypotension promptly reestablished normal vessel caliber.

In vivo microscopy. Examination of the liver edge in the normal rat revealed a) portal venules approximately 30 to 40 micra in diameter, b) sinusoids 6 to 10 micra in diameter, and c) central and sub-lobular veins 30 to 80 micra in diameter. No hepatic arterioles were recognized. The portal venules were deep within the liver parenchyma, and flow within them was rapid without interruption or intermittency. No sphincteric action could be recognized at the tips of the smallest branches of the portal venules. Branching and intercommunicating sinusoids were observed clearly, though only infrequently could a single sinusoid be traced from origin to termination. Minute constrictions were noted frequently where sinusoids emptied into central and sublobular veins. Two or more sinusoids often joined to form a common channel before entering a collecting vein. Individual sinusoids at different moments contained flowing blood, stationary cells or else appeared empty. This intermittency of flow was associated with changes in sinusoid caliber and with what seemed to be sphincteric action at the junction of sinusoid and collecting vein. The caliber of the larger efferent vessels remained nearly constant, as did the velocity of flow within them. No arterio-venous or veno-venous anastomoses were seen.

Twenty-four rats were bled. Withdrawal of less than 0.7 cc/100 cm^2 of body surface produced no change in intrahepatic vessel caliber or blood flow. Slightly larger hemorrhage was quickly followed by blanching of the sinusoid bed. More than half of the sinusoids became bloodless and the remaining columns of cells moved slowly. Many of the open sinusoids were markedly narrowed at their junctions with the central veins. Central and sublobular veins were detectably narrowed. Sinusoid caliber and flow slowly returned toward normal if blood withdrawal stopped, constriction persisting longest in the central portion of the lobule.

Restoration of the original blood volume (15 rats) produced immediate congestion. All vessels except portal venules dilated beyond their original diameters. For some five minutes, the rate of flow remained slow, even though no site of persisting constriction was observed. Thereafter, flow gradually increased throughout the liver vasculature; the sinusoids resumed their normal size, first in the periportal areas where relative paling of the sinusoid bed could be discerned, later in the centrilobular zone. Finally, the central and sublobular veins narrowed to their resting diameter and flow again became brisk. Within 30 minutes the field regained a normal appearance in animals which remained at a stable normal arterial pressure and in those which subsequently relapsed into "irreversible" shock. The livers were not grossly congested at post-mortem examination in rats dying of irreversible hemorrhagic shock.

Discussion. There is reason to believe that, in the dog, the injury sustained by the liver during a long period of severe hemorrhagic hypotension is related to the failure of recovery after replacement transfusion(5). Intrahepatic vasoconstriction persists in the post-transfusion period and obstructs venous return from the splanchnic bed(1,2), but splanchnic obstruction is not the critical effect for an Eck-fistula does not protect the dog in hemorrhagic shock(6). Pharmacologic efforts have failed to release intrahepatic vasoconstriction so as to improve blood flow through the liver in "irreversible" shock(10).

In the rat, good flow within the hepatic sinusoids is restored by transfusion even if there is subsequent relapse into irreversibility. In most instances, the portal pressure is not elevated after blood replacement or after additional transfusions. The post-transfusion congestion which persists in the dog is transient in the rat. In addition, the rat's liver may be less vulnerable to anoxia than the dog's

liver, for, unlike the dog, the rat tolerates hepatic artery ligation without antibiotic protection(7,8) and, again unlike the dog, the normal rat's liver does not harbor bacteria(9).

Summary. 1. The average portal venous pressure of rats was 15 cm H_2O in the control period, 9 cm H_2O after bleeding to an arterial pressure of 45 mm Hg, and 14.5 cm H_2O immediatly after blood replacement. 2. Direct microscopy disclosed reduction in blood flow and narrowing of sinusoids, central venules and sublobular venules after bleeding from 0.7 to 1.0 cc/100 cm^2 of body surface. These changes became extreme after a hemorrhage of 2.1-2.4 cc/100 cm^2. Replacement transfusion after 3 hours of shock restored normal vessel caliber and blood flow, whether recovery followed or not.

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Embryonic Development in Turkey Eggs Laid 60-224 Days Following Removal of Males. (20202)

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Turkey spermatozoa remain viable within the body of the female for a far longer time than those of chickens. Fertile turkey eggs have been obtained and even poults hatched from eggs laid more than 50 days after removal of the male or following a single artificial insemination(1-3). An occasional fertile egg was encountered as long as 70 days after a single insemination(4).

Routine fertility checks of segregated turkey females were made by breaking incubated eggs laid by these hens prior to the time the birds were to be used in later matings. During the course of these fertility checks, eggs were encountered showing what appeared to be a delayed and retarded type of embryonic development. Furthermore, eggs showing these forms continued to be laid beyond the expected time limit recorded previously for the duration of fertility in turkeys. These observations made it desirable to study in greater detail the factor or factors responsible for eggs showing development of this type.

Materials and Methods. A group of 30 Beltsville Small White turkey hens, 32 to 38 weeks of age were segregated from males, on Jan. 21, 1952. Prior to the time of housing these 30 hens had been with sexually mature males of their own age but none of the females had produced any eggs. Every precaution was taken to assure that no male came in contact with these females after January 21. The first egg was laid by this group of 30 hens on March 18, 1952, 54 days after the removal of the males. All eggs produced thereafter were saved for incubation and were stored in a refrigerated room maintained at a temperature of about 55°F. The relative humidity averaged about 80%. The eggs were placed in an incubator each week and each setting of eggs was incubated for a period of 7-11 days. Upon removal of the eggs from the incubator they were broken and examined macroscopically for evidences of development. The

TABLE I. Incubation Data on Eggs Laid by 30 Beltsville White Turkey Hens at Various Periods Following Removal of Males.

Month eggs laid	No. hens	Time after removal of males, days	Total No. eggs set	No. eggs showing embryonic development	%
Mar.	30	54- 67	39	12	30.8
April	30	68- 97	288	52	18.1
May	29	98-128	435	65	14.9
June	29	129-158	97	20	20.6
July	3	159-189	42	4	9.5
Aug.	3	190-220	27	2	7.4
Sept.	3	221-229	6	1	16.7
Totals and avg			934	156	16.7

germinal discs of representive eggs showing embryonic development were removed, fixed in Bouin's, and sectioned later. On June 4, 1952, 29 of the original 30 hens remained. Twenty-six of these were placed in breeding pens with mature males on that date. The remaining 3 hens were transferred to wire cages where they remained isolated from males. Eggs laid by these three hens were incubated until Sept. 15, 1952 when egg production ceased.

Results. Data are presented in Table I showing the total number of turkey eggs laid each month and the number and percentage of such eggs that showed some degree of embryonic development. The development encountered was of a delayed type first becoming visible before the egg candler after 4 days of incubation in contrast to normal embryonic growth which is visible at 24 hours. Furthermore when it did occur, it continued in most cases for only a limited time. Growth of the extra-embryonic membranes usually stopped after covering an area of the yolk surface equivalent to that occupied by a normal 2-or 3-day embryo. Typical examples of this delayed and retarded type of development as the membranes appear on the yolk are shown in the photographs in Fig. 1 and 2.

In most instances no definite embryo could be detected on gross macroscopic examination. Cross sections of these embryonic areas however often showed groups of centrally located cells attempting, with various degrees of success, to form embryonic folds and organs. A number of embryos were found however where blood islands had formed and in a few instances blood vessels were in evidence.

There were a number of noticeable exceptions however where more or less normal appearing embryos developed, though retarded. These included 2 live embryos from eggs laid 78 and 94 days respectively after the females were confined to the house. In both instances, the embryos after 8 days of incubation had attained a size equivalent to a normal 4-day embryo. The heart of each of these embryos was still beating at the time the eggs were examined. In both instances there was a well-developed vascular system.

A third embryo was encountered which had attained a size equivalent to that of a normal 7-day embryo. The egg in this instance had been incubated for 11 days, and was laid 195 days after the female had last been in contact with a male. This embryo, although not alive at the time of examination, still showed a well-developed system of blood vessels as is indicated in Fig. 3. A cross sectional view of this same embryo is shown in Fig. 4 where more or less normal embryonic folds and organs are in evidence.

The incidence of these retarded forms varied considerably in the eggs laid by different hens. Twenty-eight of the original 30 hens produced one or more eggs showing delayed and retarded type of development during the period covered by this experiment.

Of those hens producing eggs with this type of retarded development, 8 individuals laid eggs having less than 10% of such forms. On the other hand, 7 others produced eggs in which 30% to 60% showed embryonic development. The average for the eggs of all birds during the entire period was 16.7%.

The percentage of eggs showing delayed

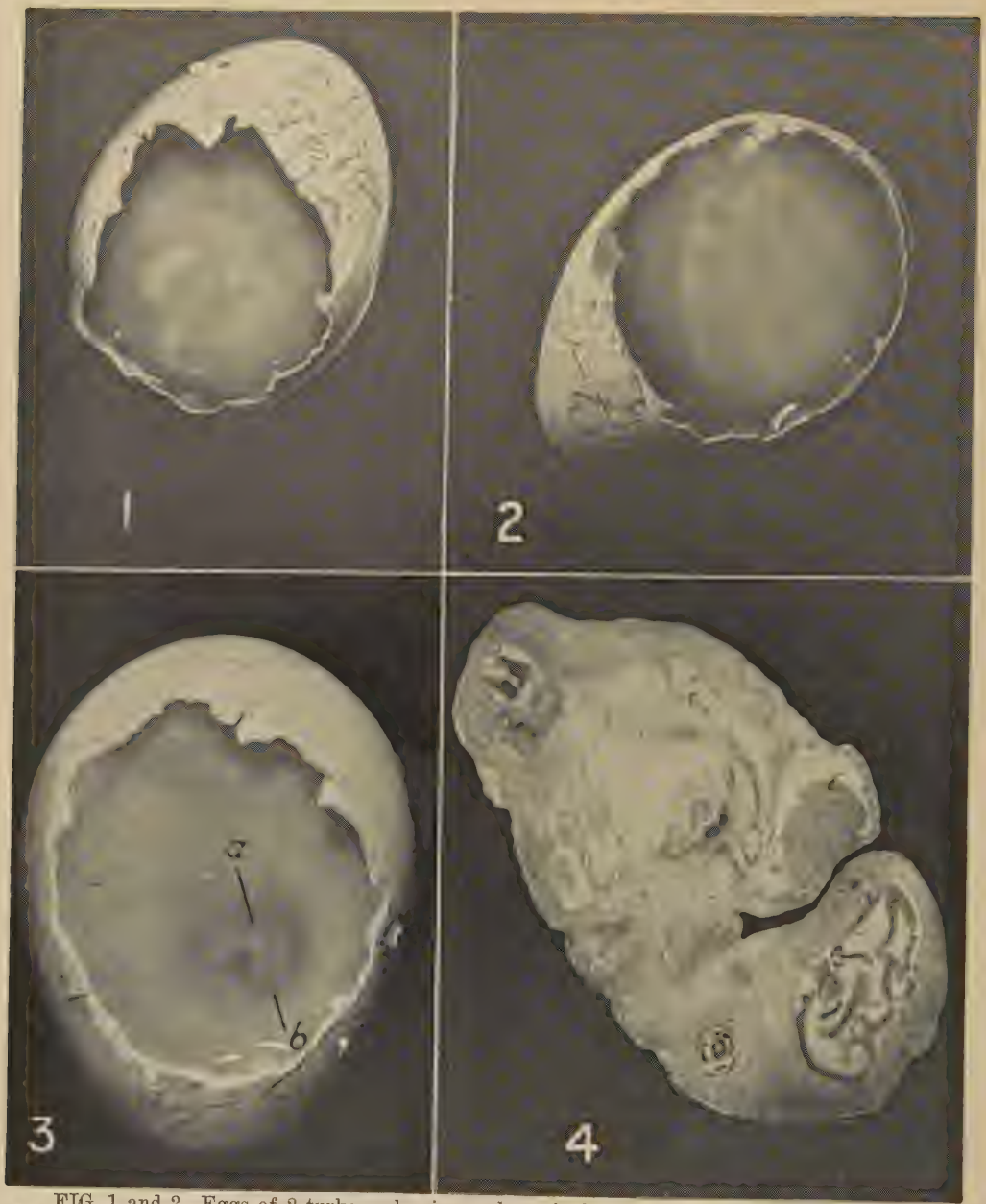

FIG. 1 and 2. Eggs of 2 turkeys showing embryonic development which occurred during a 7-day incubation period; eggs laid more than 200 days after females were isolated from males.

FIG. 3. Embryo in egg laid 195 days after isolation of the female and following an 11-day incubation period. Embryo approximately the size of normal 7-day embryo.

FIG. 4. Cross section of embryo shown in Fig. 3. Section shown is through region indicated by line ab.

embryonic development during July and August was considerably lower than that recorded for the prior months. This apparent decrease in the incidence of eggs showing these delayed forms (Table I) can be accounted for when the records of the 3 hens used for this particular portion of the experiment are examined. One of these 3 hens never produced an egg throughout the entire season that showed these retarded embryonic

forms. A second hen laid eggs averaging only 5% of these forms while the third hen produced eggs, 13.3% of which showed embryonic development during the entire season.

Discussion. It is evident from the data presented that delayed and retarded embryonic development occurred in some eggs produced by the Beltsville Small White turkeys referred to in this paper. The activating factor is uncertain in this instance since the eggs were produced by turkey hens which had at one time been in contact with sexually mature males. This much is certain however, that some of these developmental forms occurred in eggs laid by hens which had been separated from males for more than 200 days at the time the eggs were laid. This is about 3 times longer than the 70-day figure previously given (4), as the maximum duration of fertility in Broad Breasted Bronze turkeys. This would lead one to suspect, if we assume that sperm initiated this form of development, that the spermatozoa were stored for these prolonged periods in a more favorable environment than that found in the upper end of the turkey oviduct. One likely location would be within the young follicles on the ovary. It has already been shown in the case of the domestic hen that sperm are able, if deposited on the surface of the immature ovary, to enter the young ova and live there until the egg matures and in some instances to initiate development after the ovum leaves the ovary(5). Fertile chicken eggs were reported as long as 5 months after the sperm had been applied to the surface of the immature chicken ovary.

If it is concluded that sperm were not the activating factor in this instance, then it must be assumed that the egg itself, in the absence of the sperm, was activated by some other stimulus and induced to undergo some type of development. If this were true it might be a case of natural but delayed parthenogenesis in a higher order of animal. To our knowledge no case of natural parthenogenesis, to this extent has ever been reported in birds. It is true that several investigators have reported what they considered to be cells, some nucleated, in the blastodisc of newly laid infertile chicken eggs produced by non-mated and virgin females(6-10). This, however, involved at the most only a relatively few cells.

Until more critical experiments are conducted in which turkey poults of the two sexes are separated at an early age, so that the role of sperm can be ruled out, the activating factor involved must remain in doubt. Such critical experiments are not being conducted at the United States Agricultural Research Center, Beltsville, Md.

Summary. A delayed and retarded type of embryonic development occurred in 16.7% of the Beltsville Small White turkey eggs laid 54-224 days after the females were separated from males. This development in most instances could not be detected macroscopically earlier than the 4th day of incubation. The embryonic membranes usually discontinued their growth after covering an area of the yolk equal to that occupied by a normal 2- to 3-day turkey embryo. In most instances no embryo could be detected macroscopically although serial cross sections showed a definite organization on the part of centrally located cells. Two living embryos were found in eggs laid 78 and 98 days respectively after the hens were separated from the males. These embryos, after 8 days of incubation had attained a size equivalent to a normal 4-day turkey embryo. The embryonic hearts were still beating and there was a well developed vascular system in both cases. A 7-day embryo, with a well-developed vascular system was also found in an egg which had been incubated 11 days. This egg was laid 195 days after the female had been separated from males.

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Bwamba Fever Virus and Semliki Forest Virus* in Young Dogs. (20203)

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The Bwamba fever strain of virus used for this study was obtained from Dr. K. C. Smithburn of the Rockefeller Foundation Laboratories in New York. The virus was isolated from mice after the intracerebral inoculation of serum from patients. Mice develop an encephalitis after intracerebral inoculation(1).

The Semliki Forest virus (6th passage MBB26146 in serum M494744958) was also obtained from Dr. Smithburn. A viremia and encephalitis is produced in mice when inoculated with this virus by various routes. Guinea pigs, rabbits, rhesus and red tail monkeys develop the disease only after intracerebral inoculation. Chick embryos can be used to propagate Semliki Forest virus. Neutralization antibodies have been found in humans, but the virus itself has not been isolated from them(2). The experiment in young dogs was undertaken to see if they are susceptible to these 2 viruses by various routes of inoculation.

Materials and methods. Twenty Swiss albino mice (3-weeks-old) were inoculated intracerebrally with 0.03 cc of lyophilized mouse brain (Bwamba virus) of the 82nd intracerebral mouse passage. The virus received here was lyophilized mouse brain and was diluted to a 10% suspension with sterile distilled water. Twenty Swiss albino mice (3-weeks-old) were inoculated intracerebrally with 0.03 cc of lyophilized mouse brain (Semliki Forest virus) of the 6th intracerebral mouse passage using the above technic. Mice from both groups showed nervous symptoms of mild tremors and convulsions, after an incubation period of 5 to 8 days. When symptoms appeared, the mice from each group were sacrificed separately, and the brains and cords were removed aseptically. Pools of Bwamba and Semliki Forest infected mouse brains were ground with alundum, and diluted to 20% suspensions with physiological saline. The 2 suspensions were then centrifuged for 5 minutes in an angle centrifuge at 2000 r.p.m. The supernatant from each pool was used as the inoculum for initiating virus experiments in the young dogs. The virus material (Bwamba pool) was injected intracerebrally into 6 unvaccinated mice and into 6 mice which had been immunized previously with a higher dilution of Bwamba virus over a period of several weeks. After 8 days the unvaccinated mice showed symptoms of central nervous system involvement; while the immunized mice showed no nervous symptoms during a 21-day observation period. This confirmed this pool to be Bwamba fever virus. The Semliki Forest virus pool was tested in like manner (using Semliki Forest immune mice) and this pool was also confirmed to contain Semliki Forest virus. Nineteen healthy mongrel puppies of 5 to 6 weeks of age and averaging 2 to 3 lbs each were divided into 9 groups of 2 puppies each, and 1 group of 1 puppy. Two puppies of groups No. 1 to 5 were given 1 cc of a 20% suspension of Bwamba Fever virus intracerebrally, intranasally, intradermally, intraperitoneally, and intracardially. Two puppies each of groups No. 6 to 9 and 1 puppy in group No. 10 were given 1 cc of a 20% suspension of Semliki Forest virus likewise. Puppies exposed to Bwamba Fever virus were kept in an isolated room in the experimental dog kennels and puppies exposed to the Semliki Forest virus were treated in the same way. After inoculation all groups were observed twice daily for nervous symptoms characteristically found in mammals infected experimentally with these viruses.

The *results* of the Semliki Forest virus group are in Table I. When symptoms of central nervous system involvement appeared, the affected puppies were sacrificed, and their brains removed aseptically. The puppy brains were ground separately with alundum

* Approved Public Health permit to work with these viruses.

TABLE I. Response of Young Dogs to Semliki Forest Virus (Mouse-Adapted).

Route of inoculation	Exposed	Showing symptoms	Min to max* incubation period, days
Intracerebral	2	2	9–10
Intranasal	2	0	0
Intradermal	2	0	0
Intracardial	2	0	0
Intraperitoneal	1	1	7–8

* Incubation period = Time between exposure and onset of symptoms.

and diluted to a 10% suspension with physiological saline. The suspensions were then centrifuged for 5 minutes in a horizontal centrifuge at 2000 r.p.m. The supernatant from each suspension was injected intracerebrally into 6 unvaccinated mice and into 6 mice which previously had been immunized with Semliki Forest virus. After 3-4 days the unvaccinated mice showed symptoms of central nervous system involvement, while the immunized mice showed no nervous symptoms and were discarded after a 21-day observation period.

Summary. 1. A strain of Semliki Forest virus passaged intracerebrally in Swiss albino mice has been successfully transmitted intraperitoneally and intracerebrally to mongrel puppies. 2. Puppies exposed by intacerebral, intranasal, intradermal, intraperitoneal, and intracardial routes to Bwamba Fever virus and puppies exposed by intranasal, intradermal, and intracardial routes to Semliki Forest virus showed clinically no signs of nervous symptoms, and mice injected intracerebrally with brain suspensions from these puppies showed no evidence of Bwamba Fever or Semliki Forest disease.

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Production of Pituitary Tumors in Mice by Chronic Administration of a Thiouracil Derivative.* (20204)

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In the course of an investigation of the long term effects of the administration of large doses of I^{131} to mice, Gorbman(1) observed the development of tumors of the anterior lobe of the pituitary gland in those animals who survive complete destruction of the thyroid gland. Many of these pituitary tumors were very large with weights more than 100 times the normal pituitary weight. This observation was soon confirmed by subsequent studies from his laboratory(2) and by others (3,4). Two different opinions as to the mechanism resulting in the formation of these adenomas of the pituitary have been expressed. Goldberg and Chaikoff(5) found that U.S.P. thyroid added to the mice's diet in small amounts prevented the formation of pituitary adenomas following radiation thyroidectomy and concluded that the enlargement of the pituitary gland induced in the mouse by I^{131} injections results from hypothyroidism alone. Gorbman(2) also found that thyroxin injections reduced the incidence and size of the pituitary tumors expected to develop after I^{131} and that implants of normal thyroid gland which remained viable were effective in preventing formation of pituitary tumors. However, more recently Gorbman has postulated that it is not the hypothyroid state alone which causes the development of the tumors but that a factor related to the ionizing radiation itself may be an influence aggrevating or synergizing the thyroidectomy

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‡ Clinical Fellow of American Cancer Society.

in its tumorigenic acton upon the pituitary gland. He bases this opinion on the fact that in later experiments, mice which had been pretreated with a low-iodine diet and in which he was able to accomplish complete or almost complete destruction of the thyroid gland with 30 μc of I^{131} did not develop pituitary tumors; while in contrast, mice on regular diets in whom a comparable destruction of the thyroid gland was caused by 200 μc of I^{131} did develop pituitary tumors. In another experiment(6) mice on the low-iodine diet were radio-thyroidectomized with 30 μc of I^{131} and were then exposed to 545 R of whole body x-irradiation or were given an additional 170 μc of I^{131}. These animals did develop pituitary tumors.

If it is the hypothyroid state alone which causes development of these pituitary tumors, either total thyroidectomy or administration of one of the antithyroid drugs should be effective in their production. Seifter *et al.*(7) report greatly enlarged pituitaries in 2 of 3 rats killed after 2 years. One of these in a selenium-treated rat was a chromophobe adenoma, the other in a thiouracil-treated rat was a chromophobe carcinoma. Whether these tumors resulted from administration of the antithyroid drugs is open to question because a fair number of pituitary adenomas develop spontaneously in old rats. Dalton, Morris, and Dubnik(8) reporting on the long term administration of thiouracil to mice describe 2 cases on the drug for more than 464 days in which the pituitaries had an adenoma-like appearance.

Materials and methods. In the course of other experiments carried out in this laboratory, a number of male A-strain mice, 4 to 6 weeks old, were placed on a diet consisting of 0.8% propylthiouracil§ in Purina fox chow with meat meal. These mice were maintained on this diet until the last 4 were sacrificed after an interval of 534 days. In addition another group of mice of the same age and strain was set up. Each mouse of this group had a surgical thyroidectomy. This thyroidectomy was done with the aid of a dissecting microscope and at the time of surgery it was thought that all thyroid tissue had been removed.

Results. At autopsy 2 of the 4 mice on propylthiouracil had massive tumors of the pituitary gland. In one mouse the gland was about 5 mm in diameter and in the other it was approximately 3 mm in diameter. The larger of the 2 glands was hermorrhagic. In the third mouse the gland was 2 to 3 times normal size. There was no marked enlargement of the pituitary in the fourth mouse. Microscopic examination of the pituitary glands of the first two mice showed multiple adenomata of the anterior lobe composed of large chromophobe cells with large nuclei. Several large adenomata made up of chromophobe cells were also seen in the gland of the third mouse. In the fourth mouse, the anterior lobe of the pituitary appeared normal except for the fact that the chromophobe cells seemed to be diffusely enlarged and increased in number.

All 4 mice on propylthiouracil had markedly enlarged thyroid glands—the glands being about 10 times their normal size. Microscopic findings in all were similar. In some areas the glands had an alveolar structure with the follicles composed of tall cells and containing little colloid. In other areas the alveolar pattern was lost and the cells were arranged in solid cords and sheets. In all four mice there was unmistakable evidence of invasion of veins by thyroid tissue. In addition, there was invasion of the strap muscles in one mouse and of lymphatics in another. The lungs of 2 of the animals (the 2 with the smaller pituitary glands) contained multiple, small, glistening, pale pink nodules on gross examination. Microscopic examination revealed these nodules to be composed of thyroid tissue. On the basis of this evidence, a pathologic diagnosis of carcinoma of the thyroid was made in all four animals. Carcinoma of the thyroid resulting from long term administration of thiouracil derivatives has also been reported by Hall and Bielschowsky(9) in the rat and by Morris, Dalton and Green(10) in the mouse.

The pituitary glands of all of the surgically thyroidectomized mice were normal. However, residual functioning thyroid gland tissue

§ Obtained through the generosity of the Lederle Laboratories.

was found in each animal when microscopic sections of the laryngeal region were examined.

Discussion. This material is presented at this time because of the difference of opinion which has arisen regarding the pathogenesis of the pituitary tumors which follow radioiodine thyroidectomy. It seems apparent from the fact that pituitary tumors arise in mice from the hypothyroid state caused by the antithyroid drugs as well as the athyroid state caused by adequate doses of I^{131}, that the lack of circulating thyroid hormone need be the only factor present to result in the production of chromophobe tumors of the anterior lobe of the hypophysis. Surgical thyroidectomy in our hands was unsuccessful. The hypertrophy of the residual thyroid tissue remaining after operation seemed to leave the animals in a normal euthyroid state.

Summary. 1. Data are presented on 4 mice treated for 18 months with a diet to which propylthiouracil had been added. 2. Three of 4 mice had chromophobe adenomata of the anterior lobe of the pituitary gland. 3. All 4 mice had carcinomas of the thyroid gland.

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Fractionation of the Swine Follicle Stimulating Hormone. (20205)

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A number of investigators have fractionated pituitary glands for gonadotropins. Van Dyke *et al.*,(1) reported the isolation of a swine follicle stimulating hormone (FSH) which was 80-85% "pure" by electrophoresis. Greep, Van Dyke and Chow(2) earlier published a method for obtaining FSH from swine pituitaries. Li, Simpson and Evans(3) succeeded in isolating an electrophoretically homogenous FSH preparation from sheep pituitaries which was essentially free of other pituitary hormones. The authors have investigated a number of methods to accomplish the concentration of FSH from the residues obtained as a result of the acid acetone extraction of whole swine pituitaries. It was found that two alcohol fractionations of these residues give an FSH with a high biological activity which is only slightly contaminated with luteinizing hormone (LH) and thyrotropin (TSH).

Experimental. Frozen whole hog pituitaries are comminuted and extracted by a slightly modified Lyons(4) procedure. The 70-80% acid acetone insoluble fraction contains FSH with small amounts of LH and TSH.

Step I. One kg of the wet acetone insoluble fraction (30-40% solids) is suspended in 10 liters of water and rapidly neutralized with 1N NaOH to pH 8.5-9.0. After stirring for an hour at room temperature the pH is again checked and readjusted to 8.5. The mixture is then placed in a refrigerator (0° to 5°C) overnight. Slow agitation is accomplished by an electric stirrer with a rheostat. After standing overnight the extract is centrifuged and the residue discarded. Tests have shown that more than 80% of the gonadotropin is

extracted. The supernatant is then adjusted to pH 4.5 with 1N HCl and zinc acetate added to 0.02M. The pH after the addition of the zinc acetate is usually 5.2-5.6. After standing for one hour, it is centrifuged and the residue discarded. The supernatant is then chilled to 0°C and precooled 95% ethyl alcohol added slowly to a concentration of 50% by volume. The temperature is kept at 0°C or below. After standing overnight at −5°C the crude FSH is removed by centrifugation. The precipitate can then be solubilized with sodium citrate (5-6 grams in 500 ml water), dialyzed to remove most of the zinc and lyophilized. The yield varies from 12-20 g per kilogram wet residue. If further fractionation is desired the above dialysis treatment is not necessary and the precipitate can be used directly in the next step.

Step II. The Step I FSH obtained from one kg of residue is suspended in one liter of 40% alcohol containing 0.2M dibasic phosphate (adjusted to pH 7.4-7.5). The alcohol mixture was precooled to −5° to −10°C. After stirring the mixture for 4 hours at −5°C the insoluble proteins are centrifuged and the supernatant brought to an alcohol concentration of 75%. The FSH precipitates at this point and can be removed by centrifugation. After dialysis against running tap water for 12-24 hours, it is lyophilized. The yield is 5-8 g per kg of wet residue.

Step III. In order to reduce the TSH, LH and posterior pituitary contamination of the Step II FSH the following ammonium sulfate fractionation may be employed: Five grams of Step II FSH are suspended in 500 ml of 0.5 saturated ammonium sulfate solution adjusted to pH 7.4-7.5. After stirring slowly for 2-3 hours the mixture is centrifuged and the supernatant adjusted to pH 4.8-5.0 and solid ammonium sulfate added to 0.8 saturation, whereupon the FSH precipitates. It is removed, dissolved in a small amount of water, dialyzed free of salt and lyophilized. The yield is approximately 1.5-2.0 g. The 0.5 saturated ammonium sulfate (SAS) precipitate can be re-extracted with 250 ml of 0.5 SAS and treated as above. An additional 200-300 mg of FSH can be obtained in this way.

TABLE I. Biological Activity of Various Swine FSH Fractions.

	Step I	Step II	Step III
FSH*	20–40%	50–90%	100%
LH†	3–5%	3–4%	<2%
TSH (USP u/mg)	.05–.08	.05–.1	<.05
ACTH (USP u/mg)	.01	.01	<.01
Growth hormone (%)	<3	<3	<3
Prolactin (I.U./mg)	<.5	<.5	<.5
Oxytocin (USP u/mg)	.1–0.15	.1–0.15	<.02
Vasopressin (u/mg)	.05–0.1	.05–0.1	<.02
Yield (g/kg)	12–20	5–8	1.5–3.0

* Expressed as % of Armour FSH Standard (264-151 ×). This preparation has been shown to have approximately the same FSH activity as that of Li *et al.*(3) pure sheep FSH and about 1.5 times the activity of the Van Dyke(1) preparation 446 CC.

† Expressed as % of Armour LH Standard (227-80). A total dose of 15 μg of preparation 227-80 will cause, under the conditions of Greep *et al.*(7), a 100% increase in the size of the ventral prostate in hypophysectomized male rats.

Discussion. The use of the acid acetone extraction procedure makes it possible to obtain ACTH, prolactin, oxytocin, vasopressin and FSH from the same pituitary glands. The supernatant from the initial acid acetone residue contains the activities other than FSH. Growth hormone apparently is destroyed under the conditions of extraction since conventional methods of fractionation have failed to produce fractions with appreciable activity.

Recently Raben and Westermeyer(5) have shown that glacial acetic acid extraction of hog pituitaries destroys most of the FSH, TSH and LH but leaves the growth hormone activity. It may be that the essentially anhydrous conditions of the glacial acetic acid extraction may in some way protect the growth activity and destroy the FSH, TSH and LH. However, our experience has been that the best yields of FSH are obtained if the acid acetone residue is neutralized immediately.

Swine follicle stimulating hormone prepared by the above alcohol fractionation method (Steps I and II) has biological activity equivalent to or better than that reported by Van Dyke, *et al.*(1). It has approximately 75-100% of the activity of Li's sheep FSH as measured by the Chorionic Gonadotropin FSH assay method of Steelman and Pohley(6).

The contamination with LH is only slightly higher than that of Van Dyke. As set forth in Step III the LH and TSH content can be lowered further. Repeated ammonium sulfate precipitation of Step III material by the method of Van Dyke *et al.*(1) does not improve the biological activity or reduce the contaminants. As is shown in Table I the contamination with other hormones is negligible.

Summary. 1. A simple method has been developed for the recovery of FSH from residues resulting from the acid acetone extraction of swine pituitary glands. 2. The biological activity of the preparations obtained compare favorably with those of other investigators.

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Total Body Water and Blood Volume in Hereditary Obese-Hyperglycemic Syndrome of Mice.* (20206)

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In the course of an investigation on factors influencing fatty acids and cholesterol turnover in mice with the hereditary obese hyperglycemic syndrome(1), it became necessary to determine total body water and blood volume in these animals as well as in non-obese littermates. Results of these determinations present sufficient interest to justify separate publication.

Material and method. Three types of animals were used: obese and non-obese mice (age 4 to 6 months) and "young" adult obese mice (age 2 to 3 months) chosen so that their body weights would overlap with those of the large non-obese mice. Obese animals are clearly recognizable by their characteristic shape after they reach a weight of 20 to 25 g. The range for the non-obese animals was 24.5 to 33.0 g; that for the obese 31.0 to 105.4 g. In determination of total body water, deuterium dilutions were measured with a Nier Consolidated Mass Spectrometer. Weighed amounts of heavy water from .377 to .696 g were injected intraperitoneally. Tail vein samples of blood were collected in oxalated glass capillaries, sealed and kept frozen until analysis. In a series conducted to determine optimal experimental conditions, blood samples were taken at 5-minute intervals for 30 minutes, then at 30-minute intervals for 2 hours. Heavy water levels in blood were found to increase steadily during the first half hour and to remain constant during the following one and one half hours, thus indicating that equilibrium had been reached. Table I gives a typical set of values. On the basis of these results, in the main experimental series, blood samples were taken at 30-minute intervals during the 2 hours following heavy water

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TABLE I. Atoms % Excess Deuterium in Blood of Mice as a Function of Time after Heavy Water Injection.

Time (min.)	5	10	15	20	30	60	90	120
Atoms % excess	.03	.10	.16	.17	.18	.18	.19	.18

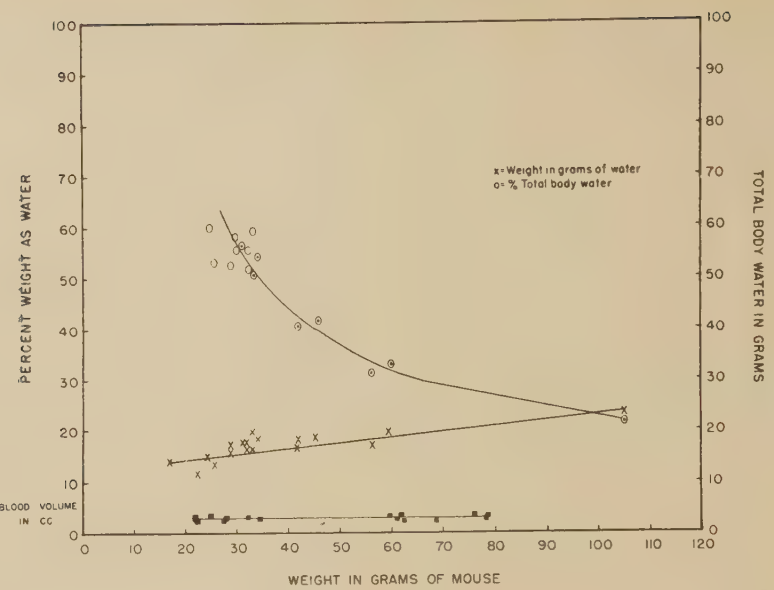

FIG. 1. Total body water, blood volume and % weight as water as a function of body weight.

injection and results averaged. Blood volume was determined by dilution of Evans blue(3).

Results and discussion. Fig. 1 gives the variation of total body water and percentage weight represented by water as a function of body weight. It is readily seen that:

1) The total body water increases only very slowly with increasing body weight. On the average, water represented only 12% of excess body weight in the older obese mice.

2) Consequently, the proportion of the weight corresponding to water decreases steadily with increasing body weight. For example, in two female littermates, one weighing 24.5 g (mouse No. 1) and the other 105.4 g (mouse No. 17), the percentage of the body weight represented by water was respectively 60% and 22%. Of the 80.9 g excess body weight carried by the obese sister only 8.9 represented water.

3) The young obese animals have a total body water and percentage body weight represented by water similar to that of the non-obese animals of the same weight. Values relating to these animals fall in the same curves as those relating to older non-obese and obese animals. Young obese animals are thus already distinguishable by their shape and by metabolic anomalies(1) from older obese at a weight which is still in the normal range and corresponds to a normal lean mass. At the same age, however, a few determinations on young non-obese animals (not included in this series) show, as expected, smaller weight, and higher weight represented by water (up to 75%), than do either obese mice of the same age or older non-obese mice.

Fat contents corresponding to the determined water content can be calculated by an empirical formula in a number of mammalian species(2,4). The extremes of percentage weight represented by fat, corresponding to mice No. 1 and No. 17 respectively (female littermates) are 18 and 70%. The median for the non-obese animals is 25%; that for the obese 45%.

Blood volume data are presented in Fig. 1. No correlation seems to exist between weight and blood volume in these animals. This result suggests that the small progressive increase in total body water, representing about 12% of the excess weight, represents the hydration of the excess adipose tissue. The lack of an increase in blood volume in obese animals may be related to the metabolic inertia of excess adipose tissue and to the absence of any increment in oxygen consumption in obese animals(5,6).

Summary. Total body water and blood volume were determined in mice with the

hereditary obese hyperglycemic syndrome and in littermate controls. Only 12% of the excess weight of obese animals was found to be represented by water. Blood volume was not increased in obese animals. The total body water and percentage of weight represented by water in young obese animals was similar to those in non-obese animals of the same weight.

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Tissue Glycogen Storage as Affected by Acetoacetate. (20207)

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After extensive studies on the effect of ketone bodies on carbohydrate metabolism, Nath and collaborators(1,2) believe that the gradual accumulation in the system of the intermediary fat metabolites, the ketone bodies, is responsible for the onset of hypoglycemia and for the gradual development of a decreased sugar tolerance in the experimental animal. These substances were reported(3) to cause an inactivation of endogenous insulin both *in vitro* as well as *in vivo*. More recently a rapid and continuing depletion of muscle and liver glycogen was found after daily injections of β-hydroxy-butyric acid had been given over a prolonged period(4). Also acetoacetate *in vitro* markedly accelerated glycogenolysis in normal rat liver slices(5). A disturbed phosphorylation was suggested as a reason for the decreased glycogen storage in these tissues. The last two studies were cited as evidence against the suggestion of Tidwell and coworkers(6,7) that the hypoglycemia after large injections of the ketone bodies might be the result of an increased glycogenesis promoted by the preferential utilization of the ketone bodies(8).

The availability of the tissues after the prolonged daily injections of acetoacetate(7) prompted us to determine the glycogen content of these muscle and liver tissues. When the glycogen values were found to be quite normal, it seemed desirable to investigate further the proposed effect of the ketone bodies on insulin action and on glycogenolysis in liver tissue, both *in vitro*. No evidence was obtained to support the contention that acetoacetate causes an inactivation of insulin or promotes glycogenolysis in the muscle or liver tissue.

Experimental. The female albino rats used in the first part of this study were those from a previous investigation(7). They had received daily intraperitoneal injections of 100 mg of acetoacetate, or an equivalent amount of propionate, per kilo of body weight the first week and these amounts were increased by 20 mg per kilo per week for 21 weeks. Similar rats which had received only the commercial stock diet for the same period were used as additional controls. At the end of this period, the rats were sacrificed by decapitation, and pieces of their livers and leg muscles were promptly removed for glycogen determinations(9). The sugar was measured after hydrolysis of the glycogen by the method of Nelson(10).

Surviving hemidiaphragms from normal unfasted rats were used to determine the effect of acetoacetate upon insulin action. The procedure of Villee and Hastings(11) was fol-

lowed with a few minor changes. The hemidiaphragms were portioned out for incubation in 4 buffer mixtures which had been prepared as follows: a) buffer mixture(12), b) buffer mixture containing 50 mg % of acetoacetate, c) the (b) mixture with 0.05 unit per ml of insulin, and d) buffer with same amount of insulin but no acetoacetate. All the buffer mixtures contained 200 mg % of glucose and were adjusted to a pH of 7.0. After incubation for 2 hours, the glycogen content of the tissues and the sugar loss from the incubating media were determined. Surviving rat *liver slices were incubated* in Krebs-Ringer-phosphate buffer solutions(13), containing either 0, 50 or 125 mg % of acetoacetate, to determine the effect of this ketone body on the glycogen of the tissue. Part of this test was repeated with the buffer used by Stadie and Zapp(12) because it contained glucose. In both cases, the liver slices were prepared according to the method of Deutsch(14). Approximately 175 mg of the tissue were incubated for 1 hour under the same conditions as were the hemidiaphragms. Again, the glycogen of the tissues and the sugar content of the media at the start and the end of this period were determined. A part of an earlier study(7) was repeated with the *following modifications*. Equivalent amounts of isotonic saline or a similar volume of isotonic glucose replaced the acetoacetate and propionate. They were injected daily in gradually increasing amounts over a period of 7 weeks. Fasting blood sugar levels were determined each week and glucose tolerances at the beginning and end of the study. Apparent *differences were tested* for by the t method of Fisher(15), and only those with a P value less than 0.01 (usually a t value of 3 or more) were considered significant.

Results and discussion. Similar amounts of glycogen were found in the livers of the rats on the stock diet and those receiving the special diets(7) along with prolonged injections of propionate or acetoacetate (Table I). This was also true of the leg muscle of these 3 groups of animals. In a previous report(7), they were shown to have had similar food intakes and growth curves with only slight alterations in their utilization of carbohydrate during the 21 weeks of the study. These results are not in agreement with those of Nath and Chakrabarti(4), who reported that smaller amounts of the ketone body injected over a shorter period caused a rapid and sustained depletion of liver and muscle glycogen.

TABLE I. Muscle and Liver Glycogen after Prolonged Propionate and Acetoacetate Injections, in 3 groups of about 10 rats each.

Body wt, g	Treatment	Glycogen, mg/g Liver	Glycogen, mg/g Muscle
244	S*	43.7 ± 6.0†	5.45 ± .57
249	Sp + propionate	48.3 ± 3.0	5.50 ± .21
255	Sp + acetoacetate	42.9 ± 4.4	5.42 ± .17

* S = stock diet; Sp = special diet.
† Stand. errors of the mean are included.

The ability of small amounts of insulin to promote glycogenesis in surviving rat diaphragm tissue(16) was used to test the effect of acetoacetate on insulin action (Table II).

The amount of glucose lost from the mixture and the glycogen of the tissue after incubation were unchanged by the presence of 50 mg % of acetoacetate. The addition of 0.05 unit per ml of insulin to these mixtures increased glycogen storage and glucose disappearance rate in both of them. Again, the glucose and glycogen changes in the 2 media were the same and there was no demonstrable effect of the ketone body upon the insulin action. The slightly, but not significantly, higher average value for muscle glycogen in the presence of acetoacetate suggests that more insulin would have sufficiently increased the rate of glucose oxidation to allow the depressing effect of the ketone body to be observed (17). In any case, there was no indication of any hindrance of the glycogenic action of the insulin of the tissue or of that added to the medium.

A similar but rapid hydrolysis of the liver glycogen (65 to 70% in 1 hour) occurred in the buffer media containing either 200 mg % or no glucose (Table III). Also, the rate of glycogenolysis was not affected by the presence of 50 or 125 mg % of acetoacetate in the absence of glucose, but it was markedly decreased by the latter amount of acetoacetate when the sugar was present. These findings were substantiated by the relative increases in the glucose of the incubation media. We are

TABLE II. Effect of Acetoacetate upon Insulin Action as Measured by Glycogen Formation. 4 groups of rats, 10-13 rats in each.

Tissue wt, mg	Buffer containing	Glucose loss from media, mg/g/hr	Tissue glycogen content, mg/g	"t" value†
151.9	Glucose	2.04 ± .15*	2.67 ± .11	
156.7	Glucose HAcAc	1.87 ± .18	2.60 ± .16	.74
156.8	Glucose insulin HAcAc	3.17 ± .17	4.29 ± .22	6.93
153.2	Glucose insulin	3.10 ± .16	3.89 ± .18	5.78

* Stand. errors of the mean are included.

† Usually a "t" value of 3 or greater indicates that difference from control value is statistically significant.

TABLE III. Liver Glycogen as Affected by Acetoacetate.

No. tests	Buffer mixture	Acetoacetate content, mg %	Increase in glucose of media, mg/g	Tissue glycogen content, mg/g	Glycogen hydrolyzed, %	"t" value
5	K*	0	23.5 ±1.0†	13.4 ± .1	65.7 ± .3	
6	K	50	25.4 ± .8	11.8 ± .5	69.8 ± 1.2	1.65
7	K	125	24.7 ± .7	13.0 ± .7	66.7 ± 1.5	.65
10	S	0	19.8 ± .8	18.8 ± .9	64.4 ± 1.7	
10	S	125	12.5 ± .4	26.0 ±1.3	50.8 ± 2.4	4.59

* K = Krebs-Ringer phosphate; S = Stadie-Zapp.

† Stand. errors of the mean are included.

not able to account for our failure to confirm the findings of Nath and Chakrabarti, who reported the amount of glycogenolysis, under conditions similar to those of the first part of Table III, to be accelerated 44 and 70% with 2 and 5 mg of acetoacetate respectively.

Similar fasting blood sugar levels and glucose tolerances were obtained after prolonged injections of isotonic saline solutions, containing sodium equivalent to that of the previously injected acetoacetate and propionate(7) or of equal volumes of isotonic glucose. As with the propionate and acetoacetate, the fasting blood sugar levels were lowered and a slightly reduced glucose tolerance was observed at the end of the injection period. Hence, these results do not support our suggestion(7) that the injected sodium might have some influence in lowering the blood sugar levels of these rats. There still remains a difference in the availability of the sodium from sodium chloride and that from the salt of an oxidizable organic acid.

An inactivation of insulin or interference in the phosphorylation process(2) by the ketone bodies is not shown by the effect of their prolonged injection upon the fasting blood sugar levels, the glucose tolerance or the glycogen content of the liver and muscle tissues. Neither does the presence of acetoacetate appear to inhibit the glycogen formation promoted by traces of insulin nor to increase glycogenolysis in liver tissue. It even seemed to decrease this process in the latter case when glucose was available to the tissue. Furthermore, the gradual onset of a hypoglycemia and a slightly reduced glucose tolerance follows not only the prolonged injection of increasing amounts of acetoacetate, but also those of propionate, sodium chloride and glucose. Hence it appears that one must conclude that any effect of the ketone bodies on carbohydrate metabolism is not likely to be the result of their inactivation of insulin.

After completion of this paper, Chari and Wertheimer(18) confirmed our results with similar concentrations of glucose, but found an increased effect of acetoacetate with decreasing amounts of glucose. Unlike their results with the diaphragm, the ketone body was found to have no effect upon the glycogen of liver slices when glucose was not added, and to aid in maintaining the liver glycogen when the sugar was present.

Summary. 1. Glycogen contents of rat liver and leg muscle were unchanged after daily injection of acetoacetate and propionate in increasing amounts for 21 weeks. 2. Ability of very small amounts of insulin to promote glycogenesis in surviving hemidiaphragms was

not inhibited by 50 mg % of acetoacetate. 3. A rapid hydrolysis of the glycogen of liver slices was unaffected by 125 mg % of acetoacetate in the buffer in the absence of glucose, but glycogenolysis was markedly decreased if both were present. 4. Acetoacetate in these concentrations does not appear to inactivate insulin, to inhibit glycogen storage in the muscle or liver tissue, or to affect appreciably the utilization of carbohydrate as measured by the glucose tolerance.

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Epinephrine Hypertensive Effects Before and After Cocaine. (20208)

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Numerous reports have indicated that cocaine increases the pressor potency of epinephrine(1-11). The questionable experimental methods employed leave this claim unsettled. The following work investigates the situation in the dog.

Procedures. Mongrel dogs were anesthetized by the intravenous injection of 30 mg of pentobarbital sodium/kg of body weight. This agent was chosen because the control blood pressure remains within physiological limits during anesthesia. Atropine sulfate, 1.0 mg/kg, was given intravenously; when necessary, additional atropine sulfate, 0.2 mg/kg/dose, was given until tetanic stimulation of the distal end of the cut left vagus failed to alter blood pressure. Blood pressure was recorded by a mercury manometer from the left common carotid artery. After securing a control record, the first dose of epinephrine was injected into the right external jugular vein. The next dose of epinephrine was injected only after the blood pressure had stabilized at approximately the preinjection level; all doses were rapidly injected, *i.e.,* within 15 seconds. Each of the 10 dogs in series 1 received repeated identical doses of epinephrine;† among the dogs, the range covered was 0.125-12.5 μg/kg. The results provide estimates of the variability of responses to such repeated doses. In series 2 each dog received the progressively increasing

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† Eastman Kodak Co., epinephrine, containing some arterenol.

TABLE I. Summary of Data on Variability of Consecutive Responses to Identical Doses of Epinephrine in 3 Dogs.

Dose in μg of base/kg	No. of doses	Preinj. blood pressure	Rise in blood pressure	Max pressure attained	Coef. of variation* (1)	(2)	(3)	(4)
.125	11	143.3 ± 2.93	7.1 ± 2.85	150.4 ± 3.75	.020	.401	.025	—
1.25	9	158.3 ± 4.20	84.1 ± 4.20	242.4 ± 7.05	.027	.050	.029	.58
12.5	7	142.6 ± 2.83	124.7 ± 2.42	267.3 ± 3.76	.158	.127	.057	.45

* (1) For preinj. blood pressure; (2) for rise in blood pressure; (3) for max b. p. attained; (4) ratio of (3) to (2).

TABLE II. Blood Pressure Responses before and after Cocaine HCl: Mean (Stand. Dev.); $P>0.1$ Except as Noted. s has 9 degrees of freedom in every case.

Epinephrine, μg/kg	Cocaine HCl, 2 mg/kg Before	Cocaine HCl, 2 mg/kg After	Cocaine HCl, 5 kg/kg Before	Cocaine HCl, 5 kg/kg After
	Max blood pressure attained in mm Hg			
Control	156.8 (14.2)	165.1 (13.9)	147.1 (14.8)	148.0 (16.7)
.01	—	—	150.5 (13.3)	148.4 (16.5)
.1	174.4 (13.9)†	187.9 (9.5)†	164.4 (13.7)	165.2 (13.7)
.5	190.6 (12.8)‡	209.2 (18.7)‡	180.7 (16.1)	195.2 (19.4)
1.0	191.6 (23.5)*	210.2 (20.0)*	184.8 (18.6)	203.1 (19.3)
5.0	—	—	232.6 (26.1)	249.8 (21.1)
10.0	250.2 (17.7)	251.0 (21.8)	280.4 (21.1)	283.9 (29.1)
	Rise in blood pressure (peak minus original control): mm Hg			
.01	—	—	3.4 (5.7)	.4 (.8)
.1	17.6 (9.0)	22.8 (14.7)	17.3 (8.1)	17.2 (11.4)
.5	33.8 (7.5)	44.1 (16.3)	33.6 (13.1)	47.2 (20.4)
1.0	45.3 (12.6)	56.5 (19.6)	44.5 (23.3)	62.2 (24.2)
5.0	—	—	85.5 (27.3)	101.8 (26.6)
10.0	123.6 (18.1)	118.8 (25.4)	103.1 (20.8)	103.0 (27.1)

* These values differ with $P<.01$.
† " " " " $.02<P<.05$.
‡ " " " " " .
For homogeneity of variance of the controls, $\chi^2 = .123$ with $P>.5$.

doses of epinephrine‡ shown in Table II; after the intravenous injection of 2 mg of cocaine HCl/kg, the doses of the hormone were repeated. The 10 dogs in series 3 received the graded doses of epinephrine‡ shown in Table II; after 5 mg of cocaine HCl, these graded doses were repeated. Epinephrine HCl was injected. Doses of the hormone are reported in μg of base/kg. The two doses of cocaine HCl did not affect blood pressure *per se*, *cf.*, Table II.

Analyses of the data follow Snedecor(12).

Results. A summary of some of the responses to repeated identical doses of epinephrine is given in Table I, including the coefficient of variation, C; results in the other seven dogs in this series were similar. The last column of this table indicates that the criterion of maximum blood pressure attained (MBPA) is more precise than is rise of pressure. The results in series I show no evidence that the first response is the largest; variance analysis, comparing the first response with the second in all 10 dogs, leads to the conclusion that such differences as exist are randomly distributed, with a probability exceeding 50%.

Table II lists the mean values of the control blood pressures, MBPA, and rises in blood pressure for the graded doses of epinephrine, both before and after the respective cocaine injections; the standard deviations are included parenthetically. Results of significance tests are given.

A dose-response curve was fitted to the data from each dog in series 2 and 3, before and again after cocaine, by the method of Brown (13). Table III contains a summary of the estimates of the parameters of the 40 dose-response curves, in so far as the estimates with probably homogeneous variances are

‡ Courtesy of Parke, Davis and Co.; this epinephrine contained less than 0.1% arterenol.

TABLE III. Summary of Parameter Estimates of the Individually Fitted Dose-Response Curves Before and After Cocaine HCl: Means and stand. dev.

Parameter estimated	Before cocaine, 2 mg/kg	After cocaine, 2 mg/kg	Before cocaine, 5 mg/kg	After cocaine, 5 mg/kg
$\overline{k}$	506.7 (539.4)	220.4 (166.6)	440.3 (547.6)	112.1 (84.5)
$\overline{b}$	3.1 (2.17)	1.8 (1.33)	3.2 (3.13)	0.9 (0.87)
$\overline{a}$	158.5 (39.0)	136.5 (28.9)	132.2 (21.9)	117.9 (27.2)

There are 9 degrees of freedom in every case. P exceeds 5% in all comparisons which are valid.

concerned(13). The administration of cocaine did not alter the estimates to any significant extent; the probability exceeds 5% for all valid comparisons.

Discussion. These results are contrary to those of most other investigators(1-13), although Melville(6) reported no marked potentiation of epinephrine effect by cocaine. All workers(6-11) who have used dogs report sensitization. Considering the variability shown in Table I, significance tests are indicated. For example, even accepting the validity of the *percentage rise* method of stating results, Tainter's data(1) yield a probability between 10-20%; this probability level is not usually considered significant. With one exception, in Tainter's data the first and smallest dose of epinephrine produced the largest response. Probably the control blood pressure in his animals, which were either pithed or urethanized, was excessively low, but the first epinephrine injection produced a more normal blood pressure, which was then maintained.

Rosenblueth(5) noted that cocaine did not alter the type of the dose-response curve for the pressor response to epinephrine except by alteration of the parameter estimates, specifically the asymptote parallel to the dosage axis. The analyses of $\overline{a}$, this asymptote, in Table III do not support this finding, as the estimates do not differ significantly from their previous values after either dose of cocaine. Neither do the analyses of the MBPA, in Table III, before and after cocaine, bear out Rosenblueth's report(5) that the MBPA are increased by cocaine, except at the single epinephrine dosage level of 1.0 μg/kg, where the probability is less than 1%.

Conclusions. The responses to repeated identical doses of epinephrine in the same dog show marked variability. In general, cocaine does not sensitize the dog to the hypertensive effects of epinephrine injections. Neither do the doses of cocaine used. 2 and 5 mg/kg, alter the level of the control blood pressure.

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Properties of a Serologically Active Substance from *Leptospira icterohemorrhagiae* (Wijnberg). (20209)

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Information concerning the chemical constitution of leptospires is sparse and contradictory. Failure to find discrete basophilic material within the cells(1) and the low index of refraction characteristic of the organisms led certain workers(2,3) to conclude that the leptospires do not contain nucleoproteins or nucleic acids. This is not in accord with the modern concept(4) of nucleoproteins as necessary participants in the growth and multiplication of cells.

Investigation of the chemical constitution of *Leptospira icterohemorrhagiae* (Wijnberg) in this laboratory indicates that nucleic acids are present in the organisms in appreciable amounts, and that the nucleotide material may be extracted in mixture with a serlogically specific antigen.

Experimental. Leptospira icterohemorrhagiae (Wijnberg) was propagated for 10 to 11 days at 30°C in Stuart's medium(5) enriched with 7.5% normal rabbit serum containing a small amount of hemoglobin, Seitz filtered and incubated at 56°C for 40 minutes prior to use. 200 ml aliquots of medium were seeded with a 20 ml inoculum of a 7-day culture. The leptospires were havested by centrifugation at 20,000 to 80,000 x g, washed twice and resuspended in isotonic phosphate buffered saline solution (pH 7) to 2% of the original culture volume. The average yield of organisms was 64 mg (dry weight) per liter of culture liquid. The resuspended organisms were disintegrated by the addition of an equal volume of a solution containing sodium chloride (0.1 M), sodium citrate (0.2 M) and sodium desoxycholate (0.4%)(6). After standing at room temperature for two hours, the suspension was held at 2-4°C for 18 to 24 hours. After complete disruption of the cells by this process, the suspension was shaken with one-half volume of a mixture of four parts chloroform and one part normal amyl alcohol(7). The biphasic mixture was allowed to stand for several days at 2-4°C, and then separated by low speed centrifugation. The slightly opalescent aqueous phase was carefully separated. The chloroform phase was re-extracted with a small portion of the disrupting fluid and the aqueous extracts combined. The preparation at this stage was designated Fraction 1 and after further purification by three more consecutive extractions with the chloroform mixture was labelled Fraction 1-A. The preparations were dialyzed in Visking cellophane bags against 0.15 M NaCl solution or phosphate buffered saline (pH 7.3), for several days and were reduced in volume by pervaporation. The final volume after dialysis was 3 to 7% of the original volume of the culture. To 25 ml of a preparation of Fraction 1 containing 0.83 mg desoxypentosenucleic acid and 0.55 mg pentose, as d(-) ribose, was added $MgCl_2$ to a concentration of 0.024 M, and 0.5 and 0.2 mg crystalline ribo-and desoxyribonuclease,* respectively. The reaction mixture was incubated at 37°C for 4 hours. The product was transferred to a Visking cellophane bag and then dialyzed in the cold against frequent changes of 0.15 M NaCl solution. The final product contained 0.50 mg pentose. The products were tested as complement-fixing antigens against known hyperimmune rabbit sera by a procedure described by Kolmer, *et al.*,(8), employing one-half volumes. *Total solids* were determined by drying aliquots to constant weight at 95-105°C. The dry weight of the cells and extracted material was estimated as the difference between the weights of the total solids in the preparations and in aliquots of the corresponding diluents. *Total nitrogen* determinations were done by a modified micro-Kjeldahl procedure using a K_2SO_4 –$CuSO_4$ digestion catalyst. *Total pentose* was determined spectrophotometrically with the orcinol re-

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TABLE I. Yield and Composition of Fraction 1 of *Leptospira icterohemorrhagiae* (Wijnberg).*

Preparation	Starting leptospirae: Dry wt of cells	Starting leptospirae: N.	Starting leptospirae: Yield solids	Fraction 1: N.	Fraction 1: P.	Fraction 1: Pentose† (as d-ribose)	Fraction 1: DNA‡
	mg						
7	48.6	5.0	23.4	2.2		1.5	1.2
9	123.0	8.0	34.1	2.1		2.2	1.4
10	115.5	6.4	73.1	2.1		2.8	2.2
13	138.6	10.2	54.7	3.3		4.5	4.2
14	192.7	7.5	38.3	3.2		4.3	2.0
15	272.0	20.4	97.4	6.1		8.1	7.8
16	554.2	36.5	120.0	8.4	2.1	10.0	8.2
Total	1444.6	94.0	441.0	27.4		33.4	27.0
% of dry wt		6.51	30.52	1.90		2.31	1.87
% of Fraction 1				6.23		7.58	6.15

* The non-dialyzable leptospiral substance is negative to biuret and ninhydrin tests but gives positive Molisch reaction.

† The colored reaction product of Orcinol-HCl-$FeCl_3$ reagent with Fraction 1 measured spectrophotometrically.

‡ Desoxypentosenucleic acid estimated by a modified procedure of Dische's diphenylamine reaction.

agent of Kerr and Seraidarian(9), using d(-) ribose as the reference standard. Desoxypentosenucleic acid (DNA) was estimated by an adaptation of the Dische diphenylamine reaction(10). *Phosphorus* determinations were made by an adaptation of the method of Gomori(11). The Beckman *DU Spectrophotometer* was used to obtain ultraviolet absorption spectra of the preparations.

Results. Seven preparations of *L. icterohemorrhagiae* (Wijnberg) yielded 1.4446 g dry weight of cells. The total nitrogen content of the leptospires was 94.0 mg or 6.5% (by weight). Fraction 1 contained about 30% of the total solids of the organisms and included an appreciable quantity of a non-dialyzable polynucleotide moiety. The chemical constitution of Fraction 1 is presented in Table I. The non-dialyzable substance containing the active principle from *L. icterohemorrhagiae* shows pronounced serological reactivity with homologous hyperimmune rabbit serum. Cross-reactions of a reduced order are noted in tests with antiserum for *Leptospira canicola*. Serological cross-reactivity with *Leptospira bataviae* antiserum is negligible. Representative data of two preparations were obtained by semi-quantitative complement-fixation titrations and are summarized in Table II. Results of a typical titration are shown in Table III. In some of the tests conducted a well defined zone of inhibition was noted in the region of antigen insufficiency.

TABLE II. Complement-Fixation End-Points of Fraction 1 of *Leptospira icterohemorrhagiae* (Wijnberg).

Antigen diluted 1:	Hyperimmune rabbit sera employed*: *L. icterohemorrhagiae*	*L. canicola*	*L. bataviae*
Preparation 14	Complement-fixation end-points		
1	>650	200	20
2.5	>650	80	10
5	>650	40	1
10	>650	40	1
20	500	10	1
40	1†	10	0
80	0‡	0	0
Preparation 16			
1 §	960	960	960
2.5§	960	480	40
5	960	240	30
10	960	120	15
20	320	30	1
40	160	1	1
80	15	1	0

* Figures represent reciprocals of highest dilutions of antisera reacting with corresponding amounts of the antigen.

† 2+ to 4+ complement-fixation reaction with undiluted antiserum, but complete or nearly complete hemolysis in the next serum dilution tested (1:10).

‡ Complete or nearly complete hemolysis on testing with undiluted antiserum.

§ Slightly anti-complementary at concentrations of antigen indicated.

Enzymatic hydrolysis of Fraction 1 with crystalline ribo-and desoxyribonuclease alters the ultraviolet absorption spectrum, but does

TABLE III. Titration of Fraction 1 of *L. icterohemorrhagiae* with Homologous Hyperimmune Serum.*

Fraction 1 diluted 1:	Homologous hyperimmune serum diluted 1: 1	10	20	40	80	160	320	640
1	4+	4+	4+	4+	4+	4+	2+	—
2.5	4+	4+	4+	4+	4+	4+	4+	—
5	4+	4+	4+	4+	4+	4+	4+†	—
10	3+	3+	4+	4+	4+	4+	4+	—
20	2+	4+	4+	4+	4+	3+	—	—
40	—	2+	2+	3+	±	—	—	—
80	—	—	—	—	—	—	—	—

* According to the method of Kolmer, *et al.*(8).
† Optimal reactivity at a concentration of antigen corresponding to 0.0026 mg of pentose, a dilution of 1 to 380000.

not entirely degrade the polynucleotides. The immunologically-reactive component retains its total activity and remains non-dialyzable. The active principle resists the proteolytic action of trypsin. It is resistant to the action of hemicellulase. After heating the product at 100°C for one hour, there is no appreciable reduction or alteration in immunological activity. Fraction 1 affords negative biuret and ninhydrin tests, but a positive Molisch reaction.

Ultraviolet absorption spectra of Fractions 1 and 1-A (Figure 1) show absorption maxima in the region of 258 millimicrons which is a characteristic property of nucleoproteins and nucleic acids. The absorption spectrum of Fraction 1 resulting from hydrolysis with a mixture of crystalline ribo- and desoxyribonuclease is shown in Fig. 2.

Discussion. The rationale for this study is based on the strong evidence of the association of nucleic acids with dynamic aspects of bio-synthesis of type-specific polysaccharide antigens and properties of virulence of certain microorganisms(12). The procedure applied to the viable cells of *L. icterohemorrhagiae* was selected for use because it involves a type of cellular disintegration which apparently causes negligible damage, if any, to highly polymerized, viscous forms of desoxypentosenucleic acid. The method possesses a distinct advantage. It avoids the dangers of degrading native cellular constituents of the leptospires as might be expected from heating or hydrolysis by strong alkaline and acid reagents. Every effort has been made not to damage the thermostable, serologically active antigen(s) inherent in the living leptospires. Fraction 1 contains, in addition to the active principle, a soluble and non-dialyzable polynucleotide moiety. Whether the immunologically-reactive sub-

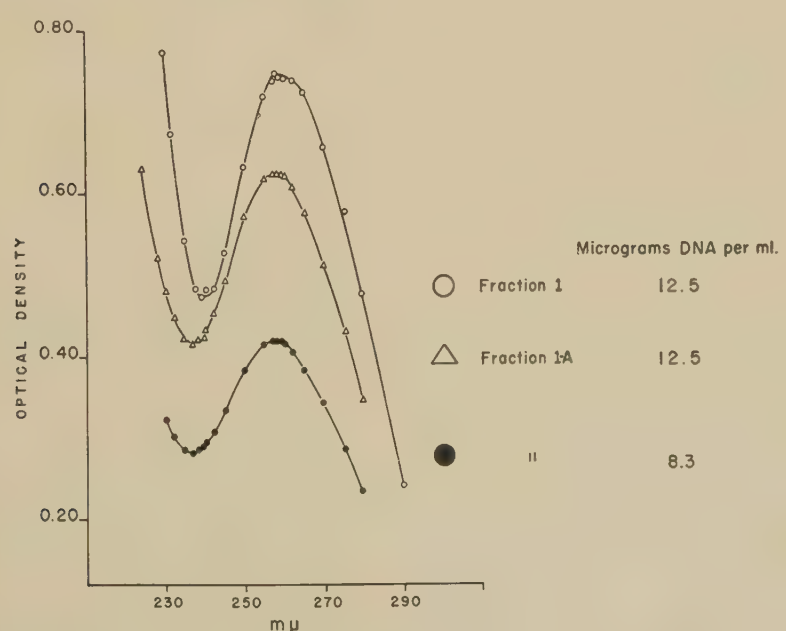

FIG. 1. Ultraviolet absorption spectrum of polynucleotide from *Leptospira icterohemorrhagiae* (Wijnberg).

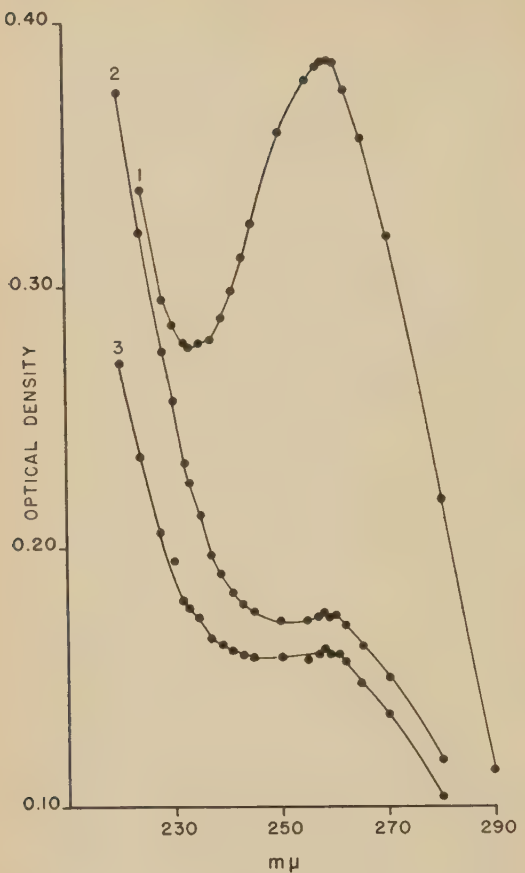

FIG. 2. Ultraviolet absorption spectrum of Fraction 1 of *Leptospira icterohemorrhagiae* before and after hydrolysis with a mixture of crystalline ribo- and desoxyribonuclease. Curve 1. Untreated material at a concentration of 11.1 μg of DNA/ml. Curve 2. Same material after 4 hr hydrolysis with the mixture of the enzymes. Curve 3. The second curve minus the optical density of the enzyme blank. E_o is based on 0.15 M NaCl solution. The complement-fixation antigen was unaffected and reacted at a concentration as low as 0.002 mg pentose/ml, a dilution of 1 to 500000.

stance and polynucleotide exists as a mixture or a chemically-bound complex has not been clearly established. Since crystalline nucleases fail to inactivate the immunological principle, it would appear likely that the polynucleotide is not the determinant of activity of Fraction 1 of *L. icterohemorrhagiae*. The active principle of Fraction 1 is resistant to digestion with trypsin. The latter fact, supported by the failure to react in the biuret test and in a sensitive test like the ninhydrin reaction, virtually indicates that the active substance is not protein.

Reaction products of orcinol and Dische diphenylamine reagents with Fraction 1 indicate the co-existence of pentoses and desoxypentose-nucleic acid.† Enzymatic treatment of the polynucleotide substance singly or with a mixture of crystalline ribo- and desoxyribonuclease is accompanied by a marked decrease in absorption at 258 mμ, thus qualitatively confirming the presence of both types of polynucleotide. Since the cells were grown on a medium devoid of nucleotides and were thoroughly washed prior to extraction, it may be concluded on the basis of the evidence presented that the organism of *L. icterohemorrhagiae* contains substantial amounts of polynucleotide material.

Pentose may be a constituent of the determinant of serological reactivity. Concentrations of 5.5 to 11.1 μg of pentose, as (d-) ribose, per ml of antigen (of which 0.25 ml is employed in the complement-fixation test) have repeatedly given maximal activity with homologous hyperimmune serum.

The presence of immunologically-reactive nucleoproteins and serologically-active pentose-associated polysaccharides is not a rarity among bacteria(13-17). The leptospires do not appear to differ greatly from other bacteria in this respect, a fact which may have some bearing on their disputed classification(3). The chemical complexity of the leptospires was noted at an early date by Noguchi(18). The latter author clearly established the existence of distinct serological types in this group of spirochaetes.

The chemical nature and properties of the determinant of immunological reactivity associated with Fraction 1 of *L. icterohemorrhagiae* and similar fractions from other serotypes of leptospires are now under study in this laboratory.

Summary. Suspensions of viable cells of *Leptospira icterohemorrhagiae* (Wijnberg) were disrupted with an equal volume of a solution containing 0.1 M NaCl, 0.2 M sodium citrate, and 0.4% sodium desoxycholate. An immunologically- reactive, non-dialyzable sub-

† A small amount of thymine was obtained out of impure starting material and was identified by chromatographic and spectrophotometric analyses.

stance (Fraction 1) was prepared by a procedure which avoided heating and use of alkalis and acids. The active principle was isolated in mixture with a non-dialyzable polynucleotide moiety. The organism of *L. icterohemorrhagiae* contained an appreciable amount of the polynucleotide substance and was composed of pentose—as well as desoxypentosenucleic acids. Crystalline ribo- and desoxyribonuclease altered the absorption spectrum of the nucleotide, but failed to inactivate the antigen. On the basis of the latter evidence it appeared likely that the polynucleotide was not the determinant of immunological reactivity. The active principle resisted the action of trypsin, hemicellulase and was not destroyed by heating for 1 hour at 100°C.

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Effect of Pantothenate Deficiency on Synthesis of Adrenal Cholesterol Following Stress.* (20210)

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The occurrence of pathological changes in the adrenal cortex of young rats maintained on diets deficient in pantothenic acid is well established(1-3). The functional capacity of the adrenal cortex is also impaired in this deficiency as indicated by the absence of a lymphopenia following swimming or ACTH (4,5), the absence of an eosinopenia following epinephrine or ACTH(5), and a decrease in glycogen deposition in the liver during fasting or anoxia(6,7). It has been inferred that this functional impairment may be related, at least in part, to low levels of adrenal steroids in the

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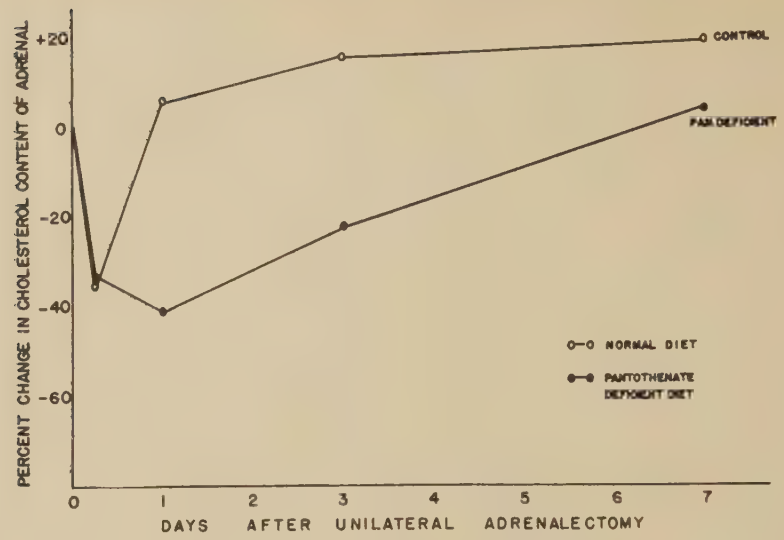

FIG. 1. Effect of Pantothenate Deficiency on Adrenal Cholesterol Determined at Intervals of 6 hr, and 1, 3 or 7 days after unilateral adrenalectomy.

pantothenate deficient rat(8-10). The data which we are presenting confirms the decreased adrenal cholesterol observed in pantothenate deficient rats(8,9) and, in addition, demonstrates that the ability of these animals to resynthesize adrenal cholesterol following stress is impaired.

Methods. Male rats, 40 days of age and weighing about 100 g, were placed on either a normal experimental diet providing 1 mg of calcium pantothenate per 100 g of diet, or on a diet deficient in pantothenic acid, but otherwise similar. After 30 days on the diets, the left adrenal of each rat was removed and analyzed for its cholesterol content by a modification of the Schoenheimer-Sperry method (11). The rats were continued on the preoperative diet after unilateral adrenalectomy. All animals received 1% NaCl as drinking water throughout the experiment. The right adrenal was removed at intervals of 6 or 24 hours or after 3 or 7 days and its cholesterol content determined.

Results. The data on the rats at the time of unilateral adrenalectomy are summarized in Table I. The body weight of the pantothenate deficient rats averaged 110 g compared with 197 g for the controls. The absolute adrenal weights were greater in the control than in the deficient rats, but when computed in terms of body weight, the adrenals of the deficient rats were relatively larger. Total adrenal weight was computed on the assumption that at the time of the operation the weights of the 2 adrenals were the same. Adrenal cholesterol was lower in the deficient rats either in terms of its concentration in the adrenal (2.29 compared to 4.40 g %) or in terms of the absolute content per adrenal (0.28 compared to 0.71 mg). Total adrenal cholesterol per 100 g body weight averaged 0.55 mg in the deficient and 0.75 mg in the control rats. The differences between the adrenal cholesterol values in the 2 dietary groups were significant as judged by the Fisher "t" test ($p < 0.01$)(12).

Post-operative survival was good in both groups of rats. After unilateral adrenalectomy the animals on the normal diets continued to gain, whereas the deficient animals tended to lose weight. At sacrifice the right adrenals of the deficient animals were found to be visibly hemorrhagic in 21 of 25 animals. No hemorrhagic adrenals were observed in the control rats. The cholesterol content of the remaining adrenal decreased in both groups of rats immediately after unilateral adrenalectomy and returned to its original level much more promptly in the normal than in the deficient rats. The per cent difference between the cholesterol content of the right and left

TABLE I. Effect of Pantothenate Deficiency on Adrenal Weight and Cholesterol Content.

	Control diet, mean ± S.D.	Pantothenate deficient diet, mean ± S.D.
No. of rats	30	25
Body wt (g)	197 ±36	110 ±25
Wt of left adrenal (mg)	16.1 ± 2.6	13.3 ± 3.7
Total adrenal wt (mg/100 g body wt)	16.9 ± 4.0	24.8 ± 7.1
Adrenal cholesterol (g %)	4.40 ± .74	2.29 ± .90
" " (mg/adrenal)	.708± .161	.277± .104
Total adrenal cholesterol (mg/100 g body wt)	.747± .231	.553± .230

adrenal was computed for each rat and the average difference at each time interval is shown in Fig. 1. Each point represents values on 5 or more rats. This method of presentation was selected because of the extreme changes in adrenal weight and cholesterol concentration which were associated with the development of hemorrhagic adrenals in the pantothenate deficient rats. As was shown in Table I, the initial adrenal cholesterol level was significantly lower in the deficient than in the control rats.

Six hours after unilateral adrenalectomy the cholesterol content of the remaining adrenal had decreased about 35% in the rats in both diet groups. No significant change in adrenal weight occurred during this period. Twenty-four hours after unilateral adrenalectomy, the cholesterol content of the remaining adrenal in the control rats had returned to approximately the level observed in the adrenal removed previously. An increase of approximately 10% in adrenal weight had occurred during this time. In the pantothenate deficient rats, however, the cholesterol content of the right adrenal was still decreased at 24 hours. This was associated with an average increase of about 10% in adrenal weight.

By 3 days after unilateral adrenalectomy the cholesterol content of the right adrenal of the control rats had increased about 15% above the pre-operative value and the adrenal weight had increased to the same extent. In the deficient rats, at 3 days, the cholesterol content of the right adrenal was greater than at 24 hours, but had not reached the pre-operative level, the concentration ranging from 0.5 to 3.5 g %. At this time the right adrenals of the deficient rats weighed 20 to 30% more than the adrenals previously removed, and with a single exception the right adrenals of the deficient rats were visibly hemorrhagic.

By 7 days the cholesterol content of the right adrenal of the control animals was about 20% above the pre-operative level, reflecting restoration of the original cholesterol concentration and moderate hypertrophy of the gland. In the deficient animals by 7 days, the original cholesterol content was restored in about half the group and was still depressed in the remainder, the concentrations varying from 3.5 to 0.4 g %. This was associated with very marked increase in adrenal weight in some animals, ranging up to almost 400% in rats with grossly hemorrhagic glands. Low cholesterol concentrations were found in the most markedly hemorrhagic adrenals.

The difference between the response of the adrenal cholesterol in the control and pantothenate deficient rats, illustrated in Fig. 1, is a significant one as evaluated by the Analysis of Covariance(12). In this analysis the total right adrenal cholesterol was the dependent variable and the total left adrenal cholesterol the independent variable. The analysis confirms the fact that a significantly different response occurred in the two dietary groups ($p < 0.01$).

Discussion. These results support the suggestion that adequate amounts of pantothenate are necessary for the synthesis of adrenal cholesterol(10). Our data on the effect of pantothenate deficiency on the cholesterol content of the adrenal confirm the observations of other observers(8,9). In addition, our observations indicate that the ability of the pantothenate deficient rat to resynthesize adrenal cholesterol after stress is decreased, as compared to the normal animal. Adrenal cholesterol is known to be synthesized from

acetate(13), and the role of pantothenate, as part of coenzyme A, in acetate metabolism is established by the work of many observers (14-16).

Both the initial increase in relative adrenal weight by the pantothenate deficient rats and the development of hemorrhagic adrenals by such rats after unilateral adrenalectomy tend to exclude the possibility of any associated pituitary defect under these circumstances. Hemorrhagic adrenals have been produced in rats on normal diets by injections of large amounts of ACTH(17). Adrenal cortical hypertrophy, an early sign of pantothenate deficiency(3), is also a typical response to the injection of ACTH(18) and can be prevented by cortisone(10).

These results, therefore, suggest that the decreased capacity to respond to stress which has been demonstrated in pantothenate deficient rats(4-7) is related to a decreased ability of such animals to synthesize the steroid hormones of the adrenal cortex.

Summary. The adrenal cholesterol of pantothenate deficient rats was significantly depressed compared to that of control animals on a normal diet. After the stress of unilateral adrenalectomy, the cholesterol content of the remaining adrenal decreased immediately in both diet groups. By 24 hours after unilateral adrenalectomy, the adrenal cholesterol of the normal rats had returned to its initial value; in the deficient rats adrenal cholesterol remained depressed up to 7 days after the stress. The data are interpreted as evidence that the synthesis of adrenal cholesterol is decreased in pantothenate deficiency.

We are indebted to Miss Elisabeth Street for the statistical analysis of the data.

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Penicillin in Milk Replacements for Dairy Calves.*† (20211)

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Recent reports have indicated that aureomycin will increase growth rates and aid in decreasing the incidence of scours in dairy calves(1-3,5,8,10-16). Other reports indicate that terramycin may stimulate growth to 8 weeks of age(4,6,9,17). In a previous report(7) it was observed that rations supplemented with potassium penicillin resulted in decreased growth and death in dairy calves fed this antibiotic at a level of 0.5 g per 100 lb of milk replacement.

The objective of the trials presented in this report was to study the use of potassium penicillin and procaine penicillin in milk replacements for dairy calves.

Experimental procedure. Six groups of 6 Holstein bull calves each, which were similar in body weight and height at the withers at 6 days of age, were housed in individual solid-walled pens and placed at random throughout the barn so as to reduce as much as possible any positional effects. Weekly body weights and measurements of height at the withers were taken of each calf on the same day of the week and at the same time of day. Daily observations were made of the conditions of the feces. If scours persisted more than 24 hours, an 8 g dose of sulfathalidine was administered orally, followed by an additional 4 g dose at each of the 2 next successive feedings. *Group I* (control) was fed the following milk replacement formula: 50 lb dried skimmilk, 10 lb dried whey, 15 lb distillers' dried corn solubles, 10 lb soluble blood flour, 7 lb dextrose, 5 lb oat flour, 0.5 lb vit. A and D concentrate (4,000 U.S.P. units and 500 U.S.P. units per gram) 0.5 lb trace mineral elements and 2 lb dicalcium phosphate. During the milk replacement feeding period the control replacement was supplemented as follows: Group I (control) received no antibiotic supplement, Group II received 0.5 g potassium-penicillin; Group III received 0.1 g procaine-penicillin; Group IV received 0.3 g procaine-penicillin; Group V received 0.9 g procaine-penicillin; Group VI received 2.7 g procaine-penicillin per 100 lb milk replacement, respectively. The *milk replacements* were dissolved in water at 100°F and were fed from open pails placed in the concentrate box located 16 in. above the floor of the pen. The rate of feeding (twice daily) was: first through 5th day—dam's milk; 6th through 10th day—2 lb whole milk, 0.2 lb milk replacement, 2 lb water; 11th through 28th day—0.5 lb milk replacement and 5 lb water; 29th through 42nd day—0.6 lb milk replacement and 6 lb water; 43rd through 49th day—0.7 lb milk replacement, 7 lb water; and 50th through 56th day—(once daily) 0.7 lb milk replacement, 7 lb water. All groups of calves were fed *ad libitum* a good quality timothy-alfalfa hay during the trial. Calf starter diet was fed *ad libitum* until each calf was able to consume the maximum of 6 lb daily for the duration of the 12-wk trial. The calf starter was prepared as follows: 416 lb ground yellow corn meal, 300 lb wheat bran, 400 lb crimped whole oats, 100 lb linseed oil meal, 300 lb soybean oil meal (44% protein), 150 lb dehydrated alfalfa meal, 100 lb cane molasses, 100 lb dried skimmilk, 100 lb distillers' dried corn solubles, 10 lb dicalcium phosphate, 10 lb ground limestone, 10 lb iodized salt, and 4 lb vit. A and D meal (1,814,544 U.S.P. units of vit. A and 226,800 U.S.P. units vit. D_2 per lb).

Experimental results. The data relative to growth expressed as gain in weight and increase in height at the withers are presented in Table I. In consideration of these data, it should be remembered that the calves received the antibiotics only until they were 8 weeks of age. It may be noted that the control group consistently gained in body weight at a more rapid rate than did any of the penicillin-fed groups. Similar results were obtained also in

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TABLE I. Effect of Potassium Penicillin and Procaine Penicillin upon Mean Daily Gains in Body Weight and Withers Height of Holstein Male Calves. 6 groups.

Supplementation/100 lb milk replacement	Gain in body wt, lb			Incr. in height at withers, cm			Incidence of scours, Da/calf
	0-4 wk	0-8 wk	0-12 wk	0-4 wk	0-8 wk	0-12 wk	
Control (none)	1.13	1.39	1.63	.16	.15	.20	5.5
.5 g K.p.*	.75	.95	1.18	.14	.12	.13	15.0
.1 g p.p.	.90	1.27	1.45	.12	.12	.15	8.0
.3 g p.p.	.83	.93	1.11	.11	.13	.12	10.5
.9 g p.p.	.97	1.23	1.38	.11	.12	.14	10.6
2.7 g p.p.	.76	1.04	1.26	.10	.11	.14	10.6

* K.p. = potassium penicillin; p.p. = procaine penicillin.

TABLE II. Supplementation of Milk Replacements with Potassium Penicillin and Procaine Penicillin and a Summary of Calf Starter and Hay Consumption of Holstein Male Calves. 6 groups.

Supplementation/100 lb	Avg starter consumption/calf, lb		Starter consumed/lb gain, lb		Avg hay consumption, lb	
	0-8 wk	0-12 wk	0-8 wk	0-12 wk	0-8 wk	0-12 wk
Control (none)	80	233	1.02	1.86	32	67
.5 g K.p.*	85	232	1.58	2.55	17	50
.1 g p.p.	89	239	1.26	2.13	16	47
.3 g p.p.	86	231	1.65	2.69	23	95
.9 g p.p.	92	232	1.34	2.18	19	52
2.7 g p.p.	84	225	1.44	2.32	27	67

* K.p. = potassium penicillin; p.p. = procaine penicillin.

respect to skeletal growth as measured by height at the withers. The potassium penicillin-fed group gained in body weight at a very slow rate until they were 8 weeks of age but then grew at a rate comparable to the control group from 8 through 12 weeks of age. A similar comparison could be made between these groups in respect to growth in height at the withers to 8 weeks of age, but after the age of 8 weeks the potassium penicillin group continued to grow at a very slow rate until they were 12 weeks of age. It appears that the calves in this group (II) gained more rapidly in body weight on a relative basis than in skeletal growth during the last 4 weeks on the trial.

The groups fed procaine penicillin increased in weight, more slowly, than the controls but were superior to those receiving the potassium salt of the antibiotic, except for Group IV (0.3 g procaine penicillin/100 lb of milk replacement) during the first 8 weeks. After the calves were 8 weeks of age and no longer received their respective antibiotic, their rates of gain in body weight increased greatly, as will be noted in Table I. During this latter period the gain of the control group, however, was still superior. The calves receiving procaine penicillin grew in height at the withers at a much slower rate than their controls, as shown by the data in Table I. This was true throughout the entire 12 weeks feeding period.

No deaths occurred in any of the groups of calves used in this trial. In the previous trial (7), several deaths occurred which appeared to be related to the potassium penicillin fed.

A somewhat greater incidence of scours was found in the groups receiving penicillin (Table I). This was especially true of the calves receiving potassium penicillin. In addition, the cases of scours occurring in the calves receiving the penicillins were more severe.

The data relative to feed consumption and feed utilization efficiency are presented in Table II. During the 12 weeks' trial the control group consumed somewhat less starter and was much more efficient in terms of pounds of body weight gained per pound of starter consumed than any of the other groups studied. In general, the procaine penicillin fed groups, with the exception of one group (0.3 g procaine penicillin/100 lb replacement), were more efficient than the potassium penicillin fed group. Although variations in hay consumption were observed between groups when net energy intakes were calculated it did not

affect the relative efficiency of feed utilization as indicated by starter utilization.

Summary. 1. Feeding of 0.5 g of potassium penicillin per 100 lb of milk replacement to Holstein bull calves resulted in a decreased rate of gain in body weight and growth in height at the withers in this experiment. 2. Feeding of 0.1 g, 0.3 g, 0.9 g, and 2.7 g of procaine penicillin also resulted in decreased growth rates in terms of mean gains in body weight and mean increase of height at the withers in this trial.

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Further Studies on Effects of a Small Intestinal Microsomal Fraction Upon Transplantable Tumors.*† (20212)

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In a previous paper(1), we reported results of exposing Gardner lymphosarcoma cell suspensions to a microsomal fraction, prepared by ultracentrifugation, from small intestinal tissue of mice and rats. The active fraction was sedimented at 90,000 x g. Animals inoculated with a mixture of lymphosarcoma cells and intestinal fraction exhibited either delayed tumor development or failure of tumors to appear within 180 days following inoculation. All control animals, given tumor cell suspensions in saline, developed tumors and died within 28 days.

Results of further work are reported in this paper, including data on effects of the fraction upon two other tumors, the Ehrlich ascites tumor and Yoshida rat ascites sarcoma. Data on the general chemical nature of the fraction are presented also.

Experimental procedure. CBA mice (Strong) were employed as hosts for lymphosarcoma and the Ehrlich ascites tumor. Tissue for subcutaneous inoculation experiments with lymphosarcoma was obtained by briefly comminuting minced tumor in 5 volumes of saline and filtering through gauze. Such suspensions

* This paper is based on work performed under contract between the Atomic Energy Commission and the University of California at Los Angeles. (See also A.E.P. UCLA Report 223)

† Portions of this study were carried out in the Department of Radiology, School of Medicine, and were partially supported by a grant from the University of California cancer research funds.

TABLE I. Effect of Pre-inoculation Exposure of Tumor Cell Suspensions to Microsomal Sediment and to Organic Solvent Extracts of Inhibitory Material.

Treatment of microsomal fraction	No. of animals	Survivals	Deaths*
Cold ethanol extraction†	8	6	2
Ethanol extraction, room temperature†	27	23	4
Petroleum ether extraction from ethanol†	35	28	7
Controls, inoculum in buffer, pH 7.4†	40	0	40
Crude sediment in buffer, pH 7.4‡	30	30	0
Controls, inoculum in buffer, pH 7.4‡	30	0	30

* All deaths from uncontrolled tumor.
† Test tumor, lymphosarcoma.
‡ Test tumor, Ehrlich ascites. ½ of the animals in experimental and control groups received subcutaneous inoculations; ½ received intraperitoneal inoculations.

were mixed with equal volumes of the crude sediment in saline or buffer solution or with extracted material prepared by treating the ultracentrifugal sediment with various fat solvents. The mixtures were incubated for 5 to 10 minutes (lymphosarcoma) or 60 minutes (Ehrlich tumor) at 37.5°C and injected subcutaneously in amounts of 0.2 ml. Ehrlich tumor cells were washed 3 times in Krebs-Ringer phosphate buffer, pH 7.4, before exposure to the fraction and subsequent intraperitoneal inoculation. Cell suspensions of Ehrlich ascites tumor were prepared from 7-day-old intraperitoneal tumors.

Results. Examination of Table I indicates that a 100% positive response was elicited in control animals inoculated with either of the tumors in saline or buffer. No animals so inoculated failed to develop and eventually die from uncontrolled tumor. The small intestines of both normal and tumor-bearing animals have yielded actively inhibitory fractions, but because of the relatively crude nature of the fraction and solvent-extracted materials, quantitative studies have not been possible. The presence of inhibitory activity in fractions from intestines of tumor-bearing animals has been demonstrated by means of inoculation and respiration studies.

a. *Chemical studies.* The active material is insoluble in 0.02M sodium phosphate solution from pH 7.4 to 8.6, in glycerol and in 5% bile solution. It has been extracted from the crude ultracentrifugal sediment by 95% ethanol at room temperature and at 5°C. (Table I), and can be precipitated from alcoholic solution by the addition of 2 volumes of saline. The inhibitor is soluble in petroleum ether and in isooctane. Active fractions were prepared by treating precipitates from ethanol solution with these solvents or by direct fractionation from alcoholic solution. In both instances, evaporation of the solvent under nitrogen yielded a yellowish oily substance. The oily material, shaken vigorously with saline or buffer, formed a fairly stable emulsion which was incubated with tumor cell suspensions prior to injection into experimental animals. Further stabilization of the emulsion was achieved in certain instances by the addition of small amounts of corn oil, which has been shown to be entirely inactive with respect to any tumor-inhibiting properties. Preliminary studies on identification have shown that the active material is soluble in acetone at −10°C and not precipitated by digitonin. Further studies are now in progress using chromatographic methods.

Ultraviolet and visible spectroscopic studies on preparations of the active material in isooctane showed the presence of multiple unsaturated bonds. Whether these were present in the active fraction or in associated fats remains to be determined. However, since these studies suggested that rapid oxidation and conjugation of fatty acids were occurring, it seemed desirable to determine whether the observed effects of the inhibitory fraction were due to fat peroxides. Accordingly, experiments were undertaken in which peroxidized corn oil and peroxidized methyl linoleate replaced the usual intestinal fraction. Results demonstrated clearly that these peroxides were without inhibitory activity. Consistent with these findings on peroxides were observations made on stability. The activity of the inhibitor declines fairly rapidly with time, and samples stored at −10°C may become inactive in 10 to 14 days. This would suggest that the activity is due to a natural labile lipid and not to a breakdown or oxidation product.

TABLE II. Oxygen Consumption Studies Demonstrating Effect of Microsomal Fraction from Small Intestine upon Slices of Lymphosarcoma and upon Certain Normal Tissues of the Mouse and Rat.

Tissue	No. of cases	Exp. treatment	Qo_2 Mean	Qo_2 S.D.	t
Mouse liver	20	C*	8.92 ± .93		
	20	M*	9.17 ± .99		.82
Mouse kidney	25	C	14.37 ± 1.44		
	21	M	15.36 ± 1.37		2.38
Mouse spleen	50	C	9.81 ± 2.04		
	46	M	8.90 ± 2.08		2.16
Rat thymus	20	C	9.32 ± 1.47		
	18	M	7.19 ± 1.37		4.63
Lymphosarcoma	20	C	12.56 ± 2.19		
	30	M	7.12 ± 1.85		9.14

* C = controls; M = microsomal fraction.

b. *Respirometry studies.* Respirometry has been a highly satisfactory means for comparing the effects of the inhibitor on tumor and normal tissues. Incubations in which slices were employed were carried out in Krebs-Ringer phosphate buffer, pH 7.4; those in which cell suspensions were used were done in Krebs-Ringer phosphate buffer of the same pH fortified with 0.15% glucose and 0.01 M sodium succinate. Temperature was maintained at 37.5 ± 0.05°C.

Data for oxygen consumption values of certain normal tissues of mice and rats and for the Gardner lymphosarcoma are summarized in Table II. From these data it is apparent that with the exception of thymus and spleen, the fraction does not affect respiration of normal tissues studied, and that the effect of the fraction upon lymphosarcoma slices is much more profound than that observed for thymus and spleen. Further comparative studies of tumor cell and bone marrow cell suspensions are in progress. The use of bone marrow cell suspensions eliminates certain difficulties inherent in interpreting results of experiments with surviving tissue slices in the presence of the insoluble fraction. Results of bone marrow experiments will be reported later.

Fig. 1 demonstrates the effect of the crude microsomal fraction on oxygen consumption of the Ehrlich ascites tumor and Yoshida sarcoma. The initial stimulation of respiration of the Ehrlich tumor following addition of the fraction is characteristic of these cells in the presence of the crude inhibitor. The stimulation is less marked when the fraction has been partially purified by solvent extraction.

Discussion. The extensive list of agents (Dyer, 2) investigated for cancer-inhibiting properties contains a relatively small number of lipid substances. Isolation from normal intestine of a lipid material with *in vitro* inhibitory effects against 3 transplantable tumors is therefore of interest.

Certain features of this material deserve emphasis. It is apparently a moderately labile lipid and is insoluble in aqueous media. The latter suggests that the inhibitor is an insoluble cellular component rather than a hormone-like compound soluble in body fluids. Its action appears to be localized *in vitro* where direct contact is required for action. This insolubility may also account for its failure to control development of malignancy *in vivo,* even though it has been shown to be present in the small intestines of tumorous animals.

Summary. 1. By ultracentrifugation at 90,000 x g, a particulate fraction inhibitory to growth of transplantable lymphosarcoma,

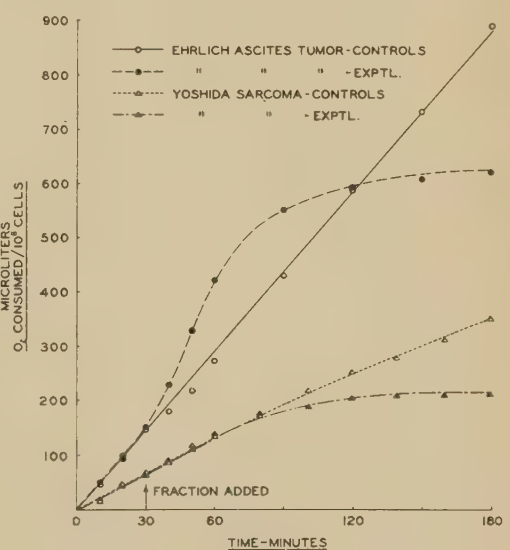

FIG. 1. Effect of crude microsomal fraction upon oxygen consumption of cell suspensions of the Ehrlich ascites tumor and Yoshida sarcoma. The fraction, suspended in phosphate buffer, was tipped into the reaction vessels 30 min. after the beginning of the experiment.

Ehrlich ascites tumor and Yoshida sarcoma has been obtained from small intestines of mice and rats. 2. Inhibitory properties of the fraction have been demonstrated by preinoculation exposure of tumor cell suspensions to the inhibitory material. 3. The intestinal fraction markedly suppresses oxygen consumption of tumor slices, slightly reduces the respiration of normal lymphoid tissues, and has no effect on respiration of other normal tissues tested; an initial respiratory stimulation was observed with ascites tumor, followed by marked depression. 4. The active component of the intestinal fraction appears to be an ethanol and petroleum ether-soluble lipid.

The authors express their appreciation to Dr. Ralph W. McKee for supplying the original Ehrlich ascites tumor animals, and to Dr. Riojun Kinosita and his staff for cooperation in the experiments with the Yoshida sarcoma.

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Effect of Stress on Diurnal Fluctuations in Eosinophils of the Laboratory Mouse.* (20213)

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Diurnal fluctuations in the number of circulating eosinophils have been described in several strains of mice(1), and in humans(2,3). Stress, and the adrenal and adrenocorticotrophic hormones cause a decrease in the number of circulating eosinophils in various animals(4,5). The purpose of this paper is to show the effect of sustained cold on the diurnal fluctuations of eosinophils in mice.

Methods and materials. In the first experiment (A) 20 normal male mice of the Jax C-57 Black Strain, obtained from Jackson Memorial Laboratory, were used. The animals were from 6 to 7 weeks old at the beginning of the experiment. They were fed a Rockland Mouse Diet, and food and water were available to the animals at all times. The mice were kept in battery jars which contained a layer of wood chips, 3 to 4 mice being placed in each jar. All of the mice were kept at room temperature for 10 days after arrival to insure full recovery from the rigors of shipment. They were then divided into two groups each containing 10 animals. One group was kept in a cold room at a temperature of 3°C except for the short periods necessary to make eosinophil counts, while the other group, which served as a control, was kept at room temperature. Eosinophil counts were made twice a week for 9 weeks using the direct count method described by Speirs and Meyer (4). Speirs-Levy eosinophil counting slides were used. A day count was made every Sunday between 1:30 and 4:00 P.M. and a night count every Thursday between 6:00 and 9:00 P.M. The interval between counts was thought long enough to permit recovery from any effects on the number of eosinophils resulting from the handling of the animals and the taking of blood. The counts were always made on the control group first and the experimental group second so that the record for each group was standard as to time of day. The actual number of cells per chamber is used as the measure of eosinophil levels of the two groups rather than the number of cells per cubic millimeter of blood since the former shows satisfactorily the relation between the eosinophil levels of the two groups, and for statistical purposes is more conservative. The cells per cu mm of blood may be obtained by multiplying the number of cells per chamber by 10. In a sec-

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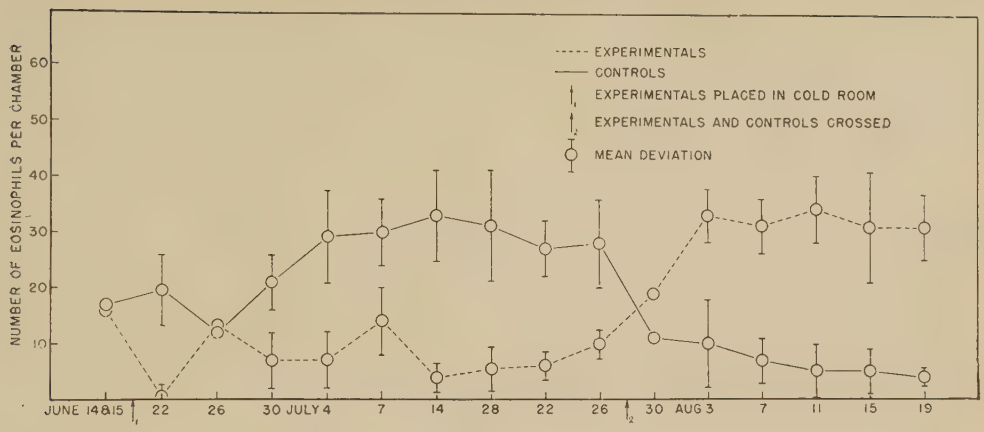

FIG. 1.

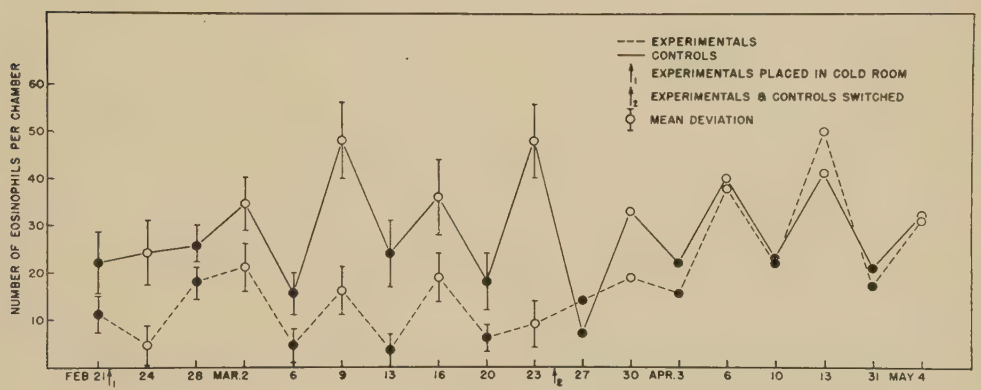

FIG. 2.

ond experiment (B) 2 groups of mice were treated in the same fashion as in experiment A but all of the eosinophil counts were made in the afternoon between 2:00 and 4:00 P.M.

Results. The results of Exp. B appear in Fig. 1 in which there is no suggestion of a marked regular fluctuation in the number of eosinophils. The low point on the graph for the control group which occurred on June 26 is correlated with extremely high temperature and humidity while the high count for the experimental group which occurred on July 9 is correlated with the rise of the temperature in the cold room due to mechanical failure of the cooling mechanism.

A graph showing fluctuations in the average number of eosinophils for the two groups in Exp. A is presented in Fig. 2. The first point on the graph represents a preliminary count made previous to placing the experimental group in the cold. Night counts are represented by dots and day counts by open circles.

After the fifth week the 2 groups in Exp. A were crossed, the experimental group being returned to room temperature and all but two animals of the control group being placed in the cold. The results appear in Fig. 2. All the mice of the control group died shortly after being placed in the cold and no counts were obtained for them. Therefore, the points on the graph for the controls after the fifth week represent the counts for only the 2 control animals not placed in the cold.

From Feb. 21 to Feb. 28 there was no apparent diurnal fluctuation of eosinophils in either group. During this period the mice were still in the process of becoming adjusted to the new situation and it was noted that they were active both during the day and the night. From March 23 to March 30 there was no

TABLE I. Differences between Eosinophil Levels during Day and Night, and between Stressed and Unstressed Mice.

	N*	Diff. ± S.E.†	t	P
I	66	13 ± 5.8	2.9	.02
II	71	10 ± 3.3	3.10	.02
III	78	25 ±11	5.10	.01
IV	64	11 ± 4.1	4.51	.01

* No. of counts.
† Difference between means ± stand. error.
I Difference between night counts of control and exp. groups.
II Difference between night and day counts for exp. group.
III Difference between night and day counts for control group.
IV Difference between night counts for exp. group before and after return to room temp.
P = Probability of a larger value of "t" by chance alone. Probability that a difference as large as that observed would occur by chance alone.

fluctuation of eosinophils among the animals of the experimental group and this is correlated with the period of adjustment immediately following their removal from the cold.

In analyzing the data 4 major categories were considered: the mean difference between night counts, of the 2 groups for the first 5 weeks; the mean difference between the night and day counts within each group from Feb. 28 to Mar. 23; and the mean difference between the counts for the experimental group before and after being returned to room temperature. The preliminary count was not used in any of these calculations. An examination of Table I shows that at the 95% confidence level there is a significant difference for each of the categories tested.

The mice kept in the cold showed a significantly smaller number of circulating eosinophils than did those kept at room temperature. That this reduction can be attributed to the cold is demonstrated by the levels shown by the experimental group after it was returned to room temperature. Examination of Fig. 2 also shows that the diurnal variation persisted in the animals in the cold and was in phase with that of the animals at room temperature. The relative degree of fluctuation is undiminished in the experimental group as may be seen by examination of the graph.

Discussion. It is clear from these results that the depressive effects of cold did not eliminate or even reduce the basic diurnal fluctuation in the number of circulating eosinophils. This suggests that the causal factors underlying the daily rhythm may operate independently of those associated with sustained stress. Though the sustained stress is apparently associated with adrenocortical function no direct evidence for the mechanism of the daily eosinophil rhythm is available. However, it has been found that the number of circulating eosinophils is higher during periods of sleep than during waking activity(2). Halberg and Ulstrom(6) have demonstrated an apparent absence of a 24-hour rhythm in the number of eosinophils in human infants under 15 months of age as evidenced by the absence of an endogenous morning eosinopenia. They suggest that this may be correlated with the absence, in infants, at this age, of the 24-hour periodicity in bodily activity characteristic of adults. They also state that the endogenous morning eosinopenia in mature man is correlated with the initiation of daily activity. Mice are characteristically more active at night than during the day and the difference in the number of circulating eosinophils between these two periods might be explained by this difference in activity. It has been demonstrated that a reduction in the number of circulating eosinophils is correlated with an increase of adrenocortical hormones(4), and that bilateral adrenalectomy eliminates the diurnal fluctuation of eosinophils in mice(7). The diurnal cycle in eosinophils might, therefore, reflect a diurnal variation in the secretion of these hormones brought about by different rates of bodily activity. These observations suggest that nocturnal activity in these experiments acted as a secondary stress superimposed upon the sustained stress of exposure to cold, and that the adrenal gland of the mouse exposed to cold is capable of responding further to the daily stress of nocturnal activity.

Summary. The well known diurnal fluctuation in the number of circulating eosinophils in mice was maintained in animals exposed to continuous cold. It is suggested that a diurnal cycle of activity is responsible for this variation and that the nocturnal activity, acting as a stress, elicits increased activity from

the adrenal cortex which is already responding to the stress of cold.

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An Efficient Apparatus for the Redistillation of Water for Biological Purposes. (20214)

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The preparation of water of sufficient purity for critical biological work, and in sufficient quantity, is a perennial problem in biological research laboratories. It is generally agreed that the output of metallic stills such as those of block tin is likely to prove unsatisfactory. Quartz is expensive and the units ordinarily available have a low output and require constant attention. Pyrex glass has proved a satisfactory substitute for most work and there are many types of Pyrex stills available which give excellent results as to quality of product. Most of them, however, are far from automatic and are generally likewise of rather low output. The type of all-Pyrex still described here has been developed after considerable experience. and has been used successfully in preparing nutrients for tissue-culture studies in a number of laboratories. It is relatively inexpensive, and once set up requires a minimum of attention.

The outfit as we use it (Fig. 1) consists of two duplicate units, each made up of 4 items, 3 of which are standard. The units are 1) a standard stove-type heater, usually a 2-burner electric stove with 3-heat burners; 2) two standard flat-bottomed 2-liter distilling flasks (Fig. 2, 3) each with 34/45 ST female joint at the top and side-arm having a 24/40 ST male joint; 3) two standard 22 cm Friedrich condensers, each with a 24/40 ST female connection at the top and standard drip point below, and 4) two constant-level inlet tubes. The inlet tubes* consist of 5 pieces (Fig. 1, 2, 3): first a) there is a feed unit consisting of a 34/45 ST male joint attached above to a 30 mm tubulation and below to one of 20 mm diameter. The lower tubulation is about 200 mm long and when inserted into the flask reaches down to the level at which the water level is to be maintained. The upper tubulation extends upward about 130 mm. Into this is fused concentrically a 10 mm tube 490 mm long so placed as to extend down 440 mm from the point of fusion, that is to about 10 mm from the bottom of the flask. and extending upward 50 mm where it ends in the socket half of an 18/9 ball joint. This forms an inlet for the water supply. From the side of the 30 mm tube a side tubulation 10 mm in diameter is brought out 40 mm, then up parallel to the concentric tube, likewise ending in an 18/9 socket joint. Connecting to this is (b) an "L" shaped 10 mm tube with ball joint at the bottom, a 280 mm vertical arm carrying a 120 mm standard Soxhlet trap, and a 130 mm horizontal arm with socket joint. Parallel to this, and connecting with the concentric tube is (c) a second "L" similar, but lacking the trap. The first "L" connects to a 100 by 100 mm "L" with a single ball joint (d) while the other connects to a 100 by 530 mm "L" with ball joint on the short arm (e). These last are inserted into a No. 12 2-hole rubber stopper. The stopper is

* Built for us by Macalaster Bicknell of Cambridge, Mass.

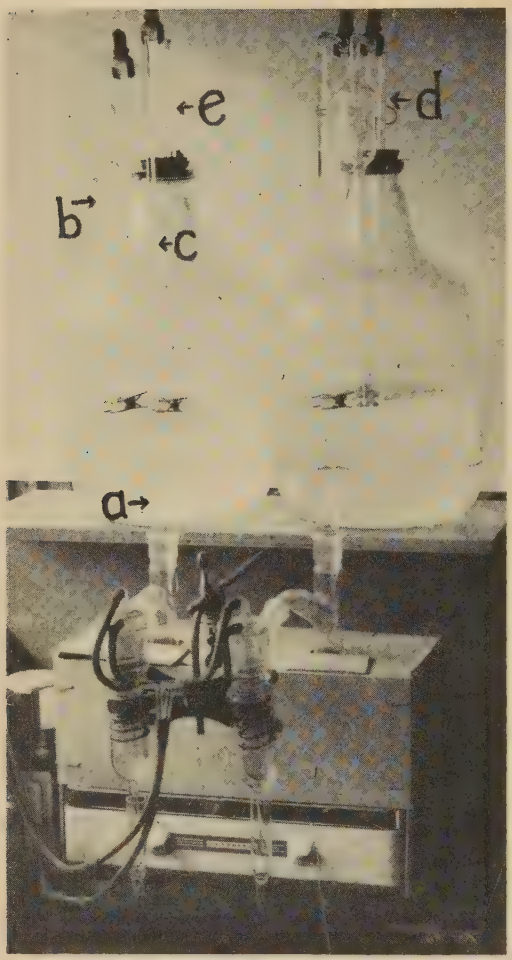

FIG. 1.

placed in a 20-liter Pyrex carboy supported at the proper height either behind or beside the distilling flask. The two flasks with their inlet tubes are set up on the 2-burner stove and the distilling flasks are surrounded by an insulating box built of asbestos board (we have found this heating unit more efficient and much less expensive than Glascol heaters). The inlet tubes are connected to the two carboys.

With the supply carboys filled with single distilled water from a tin still all joints are checked for tightness. There must be no leaks. The ball-joint between the trap (b) and the short armed "L" (d) is broken and a length of rubber hose loosely inserted toward the carboy. Blowing into the rubber hose will then force water into the other tube (c-e) and start it siphoning into the flask. The hose is then quickly removed and the ball joint closed and clamped. Water will siphon into the flask until the level rises to the end of the outer concentric tube, when the air inlet into the supply carboy is cut off and water flow ceases. As the water boils off it is maintained at this level, fresh water siphoning in whenever the water falls below the level of the tube. The trap in (b) serves to prevent excessive amounts of hot water flowing back through the air inlet with the recurrent changes in temperature of the boiling flask.

With this set up the still can be left un-

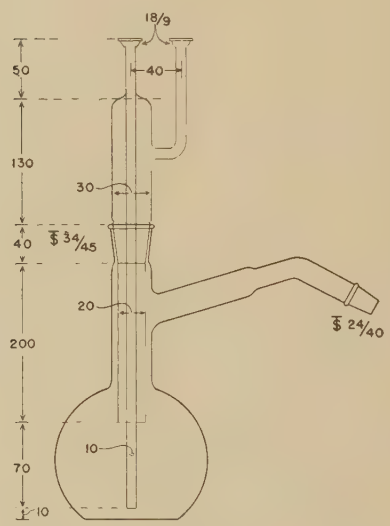

FIG. 2.

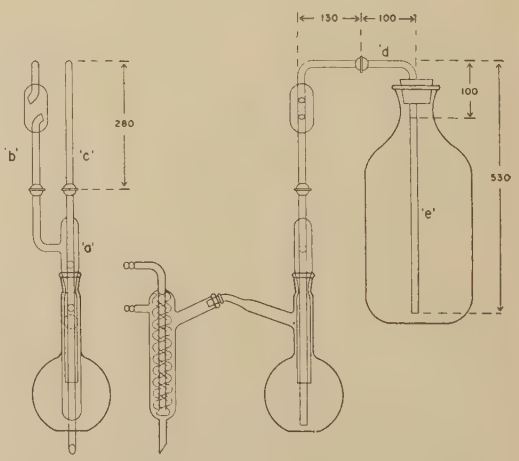

FIG. 3.

attended for long periods. If kept running continuously the supply carboys will have to be refilled about twice a week. Care must be taken they do *not* become empty, which would result in the flasks running dry and breaking, but this is the only matter that requires any attention. If the carboys can be filled directly from a central distilled water line without moving them, this can be done through a third inlet in the stoppers. In this case a stop-cock should be inserted in tube (c) so that flow of water into the distilling flask can be stopped while re-filling. Without this precaution water will continue to flow so long as the air-lock is broken in filling and the distilling flask may become flooded.

The total cost of the outfit is approximately $150. For small laboratories a single unit costing about $75 would be quite satisfactory. Some further saving can be made on the heating unit.

Two such units will distill about a liter of water an hour giving a product of high purity. With the heater, distilling flasks, tubes and supply carboys they occupy a space of 25 x 30 x 37 inches above the table.

Various refinements of this apparatus are possible. We know of at least 13 of these double units now in use. The design has proved highly satisfactory and is therefore presented with the hope that it may prove useful to others.

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Incidence of Epidermal Methylcholanthrene Tumors in Mice After Administration of Cortisone.*† (20215)

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There appears to be some relationship between the skin irritation produced by application of carcinogenic hydrocarbons and the development of the ensuing tumors(1). There is also an undoubted effect of cortisone administration in reducing the inflammatory reaction of the skin in response to the application of various irritating chemicals(2-5). It seemed of interest, therefore, to study the possible effects of cortisone administration upon both the inflammatory response and the production of epidermal tumors in the skin of mice being subjected to applications of methylcholanthrene.

Methods. Albino mice of a Swiss strain, the same age and approximately the same weight, were used. *Group I-A.* 65 mice received applications of a 0.6% (weight/volume) solution of methylcholanthrene in benzene, applied to the clipped skin of the interscapular area 3 times weekly for 6 consecutive weeks; and each received, subcutaneously, concomitant daily injections of 0.5 mg of cortisone acetate in 0.25 cc of physiologic‡ saline solution. *Group I-B* (Control). 47 mice received paintings of methylcholanthrene-benzene as in Group I-A, but daily injections of 0.25 cc of physiologic saline without cortisone. *Group II-A.* 65 mice received applications of a solution of 0.3% methylcholanthrene in carbowax 1500 (weight for weight, solution complete at 41°C) 3 times weekly for 6 consecutive weeks and concomitant daily injections of cortisone acetate, as in Exp. I-A. *Group II-B* (Control). 47 mice received applications of methylcholanthrene in carbowax as in II-A, but concomitant daily injections of physiologic saline instead of the cortisone suspension, as in Group I-B. *Tumor formation* was regarded as established when a circum-

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scribed growth of the skin was observed to persist for 3 or more weeks. (Papillomatous growth on a pedunculated base, without surrounding infiltration, suggested the absence of malignancy; nodular growth on a broad base, with central depression and indurated margins, suggested the presence of malignancy.) The *degree of irritation* was judged by visual estimation of the intensity of erythema, the amount of scaling, and the onset of epilation. In a certain number of animals of each group, skin biopsies were made at regular intervals. The present paper contains a summary of the results bearing upon the degree of inflammation and of tumor development as apparently affected by the cortisone administrations and by the trauma of the excisions for biopsy. The results of the microscopic studies and other findings will be reported fully at a later date.

Results. By comparing Group I-A with I-B and Group II-A with II-B, it was seen that in the early phases of the experiment, the cortisone administrations resulted in some reduction of the skin erythema and in a delay (averaging 4 days) of epilation and desquamation in the experiment with methylcholanthrene in benzene, as well as in a less apparent reduction of the corresponding, though weaker, responses to methylcholanthrene in carbowax. This result was not unexpected.

Contrary to our expectation, however, the results obtained with cortisone were not in the direction of reduction, but rather in the direction of higher frequency in tumor development (per number of animals): as is shown by Table I,§ 41 of the animals, which had been painted with methylcholanthrene and had received concomitant injections of cortisone, developed tumors within the first fifteen weeks of the experiment. This figure represents 69% of the "effective total" (59) of animals, *i.e.* of the number of animals alive at the time of appearance of the first tumor in the experiment. This proportion of 69% of the cortisone treated animals compares with 48% (22 of 46 animals) in the control group, I-B. Similar differences were noted in the studies

§ We wish to thank Dr. Donald Mainland, Professor of Medical Statistics, New York University College of Medicine, for his advice and evaluation of our data.

TABLE I. Incidence of Epidermal Tumors in Mice Exposed to External Applications of Methylcholanthrene, with and without Concomitant Injections of Cortisone.

Group	No. of mice:	Wk of experiment 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Totals	Total surviving mice without tumor by end of 15th wk	Total No. of mice in exp.	"Effective total No."* of mice*
I-A	M in benzene with cortisone	With signs of 1st t.g.	–	–	–	9	12	4	8	4	4	–	–	–	–	–	41	5	65	59
		Dead without signs of t.g.	1	–	3	2	3	1	2	2	–	2	2	1	–	–	19			
I-B	M in benzene	With signs of 1st t.g.	–	–	2	4	1	1	1	7	4	–	2	–	–	–	22	18	47	46
		Dead without signs of t.g.	–	1	–	–	–	–	2	2	–	–	1	–	–	1	7			
II-A	M in carbowax 1500 with cortisone	With signs of 1st t.g.	–	–	–	–	–	–	–	5	1	2	2	2	–	1	14	27	65	58
		Dead without signs of t.g.	–	1	3	–	2	1	–	1	1	1	3	7	1	1	24			
II-B	M in carbowax 1500	With signs of 1st t.g.	–	–	–	–	–	1	1	–	–	–	1	1	–	–	4	28	47	45
		Dead without signs of t.g.	1	–	–	–	1	–	3	2	1	1	–	1	1	3	1	15			

* "Effective total No. of mice" represents No. of mice alive when first tumor observed. M = methylcholanthrene; t.g. = tumor growth.

with methylcholanthrene in the carbowax vehicle. As shown in our table, 14, *i.e.* 24% of the "effective total number" of animals in Group II-A (receiving cortisone) developed tumors during the first 15 weeks of the experiment, as compared with 4 of 45 animals, or 9% in the control group, II-B.

Our results suggest that there was an additional increase in the number of tumor-bearing animals in the groups which received cortisone and in which excision-biopsies were performed at intervals. This was apparent particularly in the experiment with the benzene solution of methylcholanthrene (but was noticeable also in the experiment with the carcinogen in carbowax). Thus, in Group I-A (methylcholanthrene in benzene plus cortisone administration) 9 of 10 mice (90%) which had excisions performed, developed tumors by the end of the 9th week of the experiment—as contrasted with only 28 tumor-bearing animals (57%) among 49 mice ("effective total") which had *not* been subjected to biopsies. On the other hand, 2 (25%) of 8 surviving mice which had been subjected to biopsies in Group I-B (control experiment with methylcholanthrene in benzene, without cortisone administrations) showed tumors by the end of the 9th week, whereas 47% of the animals ("effective total") in this group which were *spared* biopsy excisions, had developed tumors at that time.

Summary. 1. Under the conditions of our experiments, the administration of cortisone to mice given external applications of methylcholanthrene in benzene or in carbowax 1500 was followed by some reduction of the early inflammatory response and by an increase in the incidence of epidermal tumor formation per number of animals, as compared with the results in the control experiments without cortisone injections. 2. Biopsy excisions performed in the methylcholanthrene-exposed skin areas appeared to increase still further the incidence of tumor formation in the mice treated with cortisone.

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Potassium Metabolism of Liver Mitochondria.* (20216)

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It has long been postulated that the active transport of potassium (K) or of other alkali metal ions such as sodium (Na) by living cells involves the intermediate formation of ion-carrier complexes(1). More recently, in various cells and tissues, analysis of the kinetics of K exchange by the use of radioactive tracer (K^{42}) has suggested that intracellular K is not homogeneous but that it may consist of separate fractions exchanging at widely differing rates(2,3). Although this finding could be interpreted as indicating the formation of an ion-carrier complex, the evidence is inconclusive and it has become essential to attempt direct observations at the sub-cellular level. Mitochondria were selected for study because aerobic phosphorylation has been implicated in K accumulation by kidney slices(4) and by retina(5); and because the capacity for catalyzing aerobic oxidation and for the aerobic generation of phosphate bond energy has been localized for the most part to this fraction of

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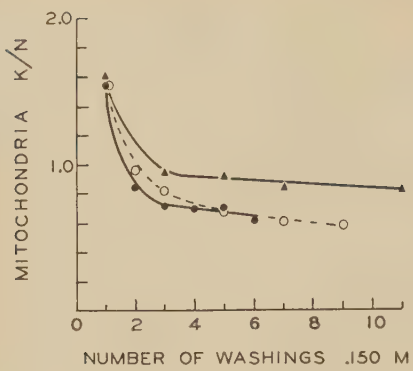

FIG. 1. Changes in mitochondrial K/N ratio following repeated washes in 0.15 M NaCl at 2°C. The 3 curves represent three different experiments. The mean K/N ratios after 3 washings were: 25 experiments with 0.15 M NaCl, 0.79 S.D. ± 0.14; 21 experiments with 0.075 M NaCl, 0.77 S.D. ± 0.06. Under the same conditions the K/N ratio of the nuclear fraction was about one-third that of the mitochondrial, but contamination of the former by the latter fraction cannot be excluded. In 8 determinations the mean K/N ratio of the intact liver was 3.3.

tissue homogenates(6). Relatively high concentrations of K have been previously reported for liver mitochondria(7) and for the protoplasmic granules of Nitella(8), but no systematic studies have been made of the metabolic characteristics of this fraction of cell K. Direct evidence has been obtained in our studies that there is "non-exchangeable"‡ K associated with liver mitochondria and that the turnover of this K in an *in vitro* system is in part dependent on aerobic metabolism.

Methods. Mitochondrial suspensions were prepared from rabbit liver by minor modifications of the technic of Lehninger(9). After homogenization in ice-cold 8.5% sucrose, the nuclear fraction was spun down in a refrigerated centrifuge at 600 x G for 5 minutes and was discarded; the supernatant was adjusted to 0.15 M NaCl by the addition of 1 M NaCl, and the mitochondrial fraction deposited by centrifugation at 1,600 x G. The mitochondria were then washed by resuspension in 10 volumes of chilled 0.075 M NaCl; similar results were obtained using 0.15 M NaCl (Fig. 1). The final mitochondrial deposit was suspended in a small amount of saline and incubated in Warburg vessels according to standard technics. Samples of fresh mitochondrial suspension and of mitochondria harvested after incubation were taken for electrolyte determination (internal standard flame photometry) and for nitrogen analysis (micro-Kjeldahl); results are expressed as mEq of electrolyte/g of mitochondrial N. The sample for electrolyte analysis was digested in conc. HNO_3 and the volume made up to 10 ml; when radioactive isotopes were used 2 ml of this dilution was taken for counting with an end-window Geiger-Muller tube. In each experiment the electrolyte content and radioactivity of the separated incubation medium were determined directly. The extent of equilibration between mitochondrial and medium electrolyte was expressed as the specific activity-ratio; this was calculated as specific activity of electrolyte in the mitochondria divided by specific activity of electrolyte in the medium. Oxygen consumption (qO_2) was calculated as μl oxygen consumed per hour per mg mitochondrial N.

Results. It was not possible to obtain a K free suspension when mitochondria were washed repeatedly with cold NaCl solution during their preparation; the mitochondrial K/N ratio of approximately 0.80 remained virtually constant between the third and tenth wash (Fig. 1). This "non-exchangeable" K of the cold mitochondria constituted at least 1.8% of the total K of rabbit liver; this is a minimal estimate based on an assumed complete recovery of the mitochondria during their preparation.

Suspensions of thrice washed *mitochondria* (M_3L) were incubated in the Warburg apparatus at 25°C. In the standard procedure each vessel contained 1 ml M_3L (5-7 mg N), 30 μM Na α-ketoglutarate, 60 μM tris (hydroxymethyl) aminomethane buffer (pH 7.4), 5 μM $MgCl_2$, 75 μM KCl, trace amounts of K^{42} and water to a final volume of 3 ml; oxygen in the gas phase. Although cytochrome-C was added in some experiments no requirement for it could be established and it was generally

‡ The expression "bound K" is avoided since it tends to have a specific physico-chemical connotation. "Non-exchangeable" throughout the present text may be taken to imply "indiffusible" or "not participating in simply physical exchange with ambient cations". This definition does not preclude exchange mediated by the expenditure of energy derived from metabolic processes.

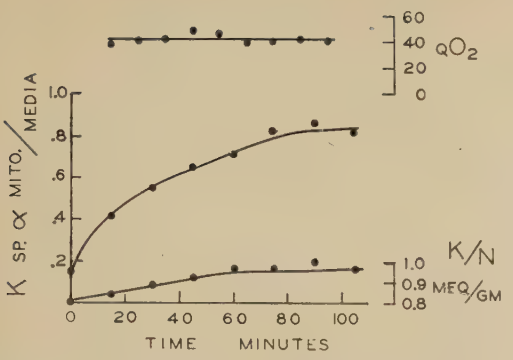

FIG. 2. Equilibration of mitochondrial K with medium K during aerobic incubation. Cup components as in standard procedure, see text.

omitted. Following incubation, flask contents were decanted into 100 ml plastic tubes, flasks rinsed with chilled 0.15 M NaCl and the mitochondria deposited by centrifugation at 2°C. Harvested material was washed 3 times with 75 ml of cold saline and then analysed for radioactivity, total K and N. *Equilibration* between mitochondrial K and the labelled K of the medium was about two-thirds complete with this system after 60 minutes incubation (Fig. 2). In 22 experiments the average K/N ratio after 60 minutes incubation was 109% of the pre-incubation value. Thus a biological steady state can reasonably be assumed even though a precise kinetic analysis cannot be made because of the time required to "harvest" the mitochondria by this technic.

The contributions made by each of the components in this test system are indicated in Table I. The highest K/N ratios and most complete K exchange were obtained under conditions which permitted aerobic oxidation of added substrate. Comparable results were obtained using α-ketoglutarate, citrate or succinate. In several experiments with α-ketoglutarate, anaerobic incubation yielded K/N ratios approximately 60% of the values obtained with the complete system, and the specific activity ratio was correspondingly reduced. Anaerobic incubation with succinate produced similar but less regular results. No systematic attempt has yet been made to study the effects of anaerobic incubation in the presence of various other substrates. It is conceivable that the K turnover found in the absence of added substrate (Table I) was associated with the oxidation of endogenous substrate but the significance of this has not been explored. Although the addition of adenosine-5-phosphate (AMP) or adenosinetriphosphate (ATP) greatly increased respiration, these compounds had no apparent effect on K metabolism and were therefore not routinely employed.

When mitochondrial K had been actively labelled with K^{42} during the period of incubation, it was found that the final radioactivity was the same whether the harvested mitochondria were washed at 2°C with 0.15 M NaCl or with 0.15 M KCl (Table II). This experiment demonstrates the exchangeability of mitochondrial K in a metabolizing system and its failure to exchange with either Na or K during the washing procedure. However, certain inconsistencies are noted when attempting to correlate K turnover with the degree of oxidative activity. During incubation, the procedures which reduced aerobic metabolism

TABLE I. Component Study. For additions see *standard procedure* in text. Mitochondrial K/N ratio was 0.86 before incubation. Incubated 60 minutes at 25°C. With the complete system the average specific activity ratio in 20 experiments was 0.65 after 60 minutes incubation.

	Values after incubation		
	qO_2	K/N	Specific activity ratio
Complete system	43	.90	.64
No α-ketoglutarate	15	.47	.70
" $MgCl_2$	40	.73	.50
" Tris buffer	42	1.05	.53
" cytochrome	39	.86	.71
" K^{39} added	41	.50	.09
" oxygen	2	.59	.46

TABLE II. Incorporation of K^{42} into Mitochondria. After paired aliquots of mitochondria had been incubated with α-ketoglutarate for 1 hour, one sample washed 3 times with 75 ml of isotonic NaCl (2°C); the other sample washed with an equal volume of KCl, which represents a thousand-fold excess of K for each wash. For discussion see text.

	Treatment of mitochondria after incubation			
	Washed in NaCl			Washed in KCl
Exp.	K/N, mEq/g	Sp. activity ratio, mito/media	K^{42}, cts/mg N	K^{42}, cts/mg N
1	1.02	.82	136	104
2	.81	.71	152	154

only partially inhibited K exchange (Table I), while virtually complete inhibition of exchange is indicated for the cold, non-metabolizing mitochondria by the type of experiment shown in Table II. An explanation for these inconsistencies is not immediately at hand.

A variety of experimental variables was examined using the incubation procedure described above. K metabolism was not detectably influenced by a change in pH between 7.2 and 7.6, but below pH 7.0 and above 8.0 the K/N ratio was reduced with a parallel depression in respiration.

Variations in osmotic pressure between 165 and 300 mOsM/1 had no significant effect on respiration or mitochondrial K. With higher osmotic pressures (produced by the addition of either NaCl or sucrose) there was a progressive fall in the K/N ratio and in the oxygen uptake, so that at 400 mOsM/1 these values were about 30% of the controls.

In the standard experiment the final K concentration in the medium was 25 mEq/1; this was selected because of the constancy of the K/N ratios and the magnitude of K exchange. When lower concentrations of K were used there was a slight reduction in the K/N ratio but a more marked effect on the exchange reaction (Table I). The effects of higher K concentrations have not yet been systematically examined.

In a system to which no $MgCl_2$ had been added, the presence of $CaCl_2$ (final concentration 3 x 10^{-4} M) reduced markedly both the rate of oxygen consumption and the level of K maintained by the mitochondria. The addition of an excess of $MgCl_2$ (final concentration 1.5 x 10^{-3} M) prevented the inhibitory effects of Ca on oxygen consumption; in some experiments depression of the K/N ratio was prevented, but this was not constant.

A surprising observation was the profound effect of inorganic orthophosphate on mitochondrial K (Fig. 3). In confirmation of the work of many others, orthophosphate was found to stimulate respiration but at the same time there was a marked lowering of the K content of the harvested mitochondria. No explanation for the latter effect can be given at present but it may be pointed out 1) that the K/N ratios fell to levels much lower than those obtained during anaerobic incubation and 2) that a maintained high level of mitochondrial K is apparently not essential for aerobic metabolism under these circumstances.

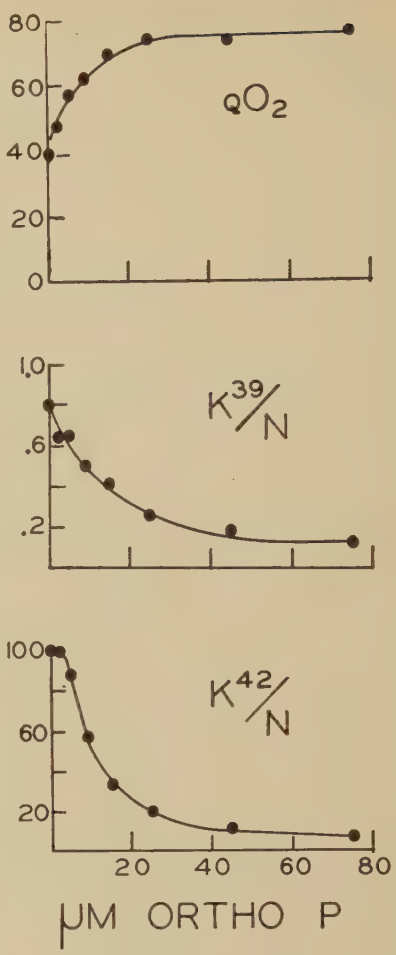

FIG. 3. Effect of orthophosphate on mitochondrial K. Standard procedure except for additions of orthophosphate, indicated as μM added per cup (final volume 3.0 ml). Osmotic pressure kept constant at 240 mOsM/1 by variations in NaCl.

Various observations, including the effects of the addition of orthophosphate described above, suggested the possibility that changes in phosphorus metabolism were specifically concerned in the turnover of mitochondrial K. Previous work had also implicated aerobic phosphorylation as a determinant in the maintenance of normal levels of tissue K. A study was made, therefore, of the effect of 2,4-dinitrophenol (DNP), measuring simultaneously the changes in both K and phosphate metabol-

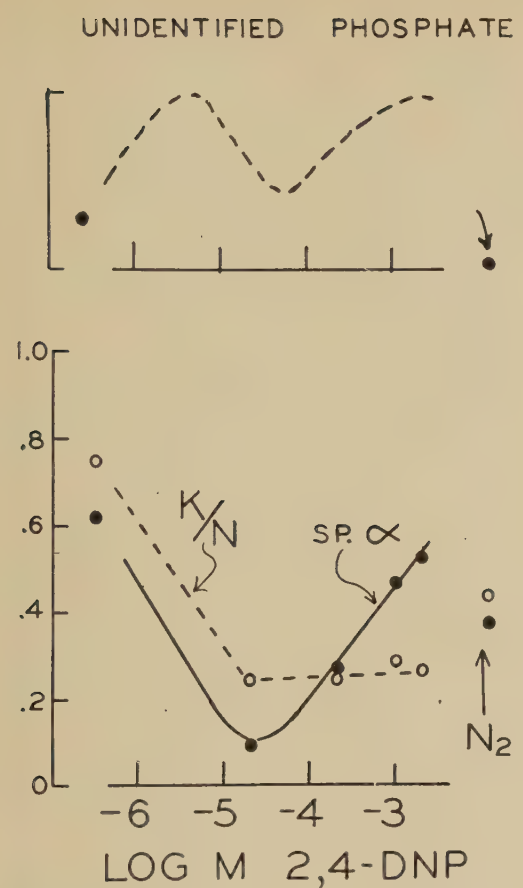

FIG. 4. Effect of DNP on mitochondrial K. The relative amount of orthophosphate esterified under these conditions is shown schematically at top. Comparison with anaerobic incubation (without DNP) is indicated by N_2.

ism. These experiments revealed a previously undescribed polyphasic effect of DNP on phosphate metabolism and on respiration; they also suggest a relationship between K exchange and the formation of certain phosphate esters. Very low and high concentrations of DNP each increased the esterification of added orthophosphate§ in the absence of added phosphate acceptors and produced a considerable augmentation of oxygen consumption; at intermediate concentrations, esterification and respiratory stimulation were minimal. The studies in phosphate metabolism will be described in detail elsewhere but the observed changes are presented schematically in Fig. 4 to permit comparison with the changes in K. Low concentrations of DNP (10^{-5} M) *increased* the amount of orthophosphate which disappeared during incubation and there was a simultaneous reduction in the concentration of mitochondrial K. Intermediate concentrations (10^{-4} M) of DNP, which are known to inhibit oxidative phosphorylation, were found to diminish further the concentration and exchange of mitochondrial K and to *decrease* orthophosphate esterification. The specific activity ratio of K in the harvested mitochondria at this concentration of DNP (10^{-4} M) was very low, much less than that obtained following incubation without oxygen. When the DNP concentration was increased to 10^{-3} M there was a marked stimulation of respiration and esterification of orthophosphate again *increased,* as did the apparent exchange of mitochondrial K. Further studies have shown that the phosphate ester thus formed is completely hydrolyzed by N HCl in 40 minutes at 100°C. The greater part of the ester formed could be recovered from the incubation medium, but separate analysis of washed mitochondria showed that incubation in the presence of 10^{-3} M DNP produced an increase in the 40-minute hydrolyzable phosphate of the actual mitochondrial particles. Since this concentration of DNP also increased the rate of exchange of mitochondrial K it seems possible that the two phenomena are related.

Further studies are now in progress to clarify the precise relationship of K to the phosphate fractions of the mitochondria. It may be noted that DNP has virtually no effect on the small amount of Na demonstrable on the mitochondria (Fig. 5). The effect on K appears to be specific.

Discussion. It is considered that the observations described warrant the conclusion that there is a fraction of cell K, associated with the mitochondria, which may be "non-exchangeable" under certain circumstances but which is capable of exchange under appropriate metabolic conditions. When metabolism

§ In a system without added orthophosphate DNP produced similar effects on respiration and phosphate esterification: the "apparent orthophosphate" estimated (Fiske and SubbaRow) in trichloracetic acid extracts of harvested mitochondria showed a completely comparable polyphasic variation.

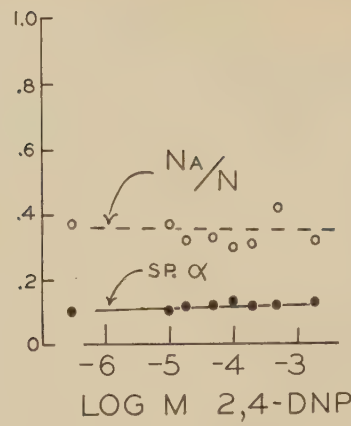

FIG. 5. Effect of DNP on mitochondrial Na. Conditions as in standard procedure except that after incubation the mitochondria were washed three times with chilled 0.15 M KCl, instead of NaCl. In 5 experiments the mean values of the control (*i.e.* without DNP) were: Na/N, 0.34; and specific activity ratio, 0.17. Under these conditions the mitochondria contain much less Na than K, and its rate of exchange is very low.

is suppressed, both by cooling to 0°C and by simultaneous dilution of substrate, this K is apparently indiffusible and unavailable for exchange with either the Na or K of the wash fluid; during active metabolism the same fraction is in a state of dynamic exchange with ambient K. Although the highest K/N ratios and the highest rates of exchange were obtained under conditions which permitted oxidation of added substrate, certain observations indicated that the behavior of mitochondrial K could not be correlated directly or solely with oxygen uptake. Most striking were the extremely low K/N ratios obtained with added orthophosphate or after incubation with DNP concentrations higher than 10^{-4} M. In both instances, although oxygen consumption increased, the K/N ratio in harvested material was lower than that observed after anaerobic incubation. In addition, there was no correlation between K turnover and the respiratory stimulation produced by AMP, nor between K/N values and the increased oxygen consumption and phosphate esterification noted with 10^{-5} M DNP.

It must be emphasized that in a study of this kind the precise results obtained may be determined largely by the chosen experimental conditions. It is, therefore, not surprising that others have reached conclusions quite different from our own. Thus, Harmon(10) concluded that the mitochondria were fully permeable to both Na and K and that accumulation did not occur even during aerobic metabolism. It may be noted that his analyses were limited to determination of radioactivity and that the washing procedures were less thorough than ours; moreover, all experiments were carried out in the presence of a concentration of orthophosphate which we have found to depress the level of mitochondrial K. While the present paper was in preparation Bartley and Davies(11) reported that the establishment of concentration gradients for a variety of ions, including K, by kidney mitochondria was dependent on metabolic activity. Both their experimental technics, and the results obtained differ from those of the present investigation. From the standpoint of technic it is noteworthy that they incubated in the presence of orthophosphate (0.01 M) and that after incubation the mitochondrial material was centrifuged down at 20°C and the deposit sampled for analysis without further washing. With this technic the mitochondria were sampled while metabolism continued and the sample must have contained diffusible potassium in the extra-mitochondrial fluid; with our own technic, metabolism was arrested and any diffusible K in extra-mitochondrial fluid was presumably replaced in the course of washing by the Na of the wash fluid. Thus, whereas we were dealing with a very few micro-equivalents of K firmly associated with the mitochondria, the K content of the samples taken by Bartley and Davies may have been largely determined by the K concentration in the original incubation medium. The K concentration used by them is not reported; but if this was high it is conceivable that the "immeasurably high" rates of K exchange which they observed represented equilibration between added K^{42} and diffusible K in the fluid *between the mitochondrial particles*. The slower turnover of the smaller amount of K *on the mitochondria* may have been completely masked. Extension and comparison of the two technics should provide further insight into the problem.

No attempt will be made to integrate all the

described phenomena; in fact, some of our observations as yet elude adequate explanation. Nonetheless, it has been demonstrated that mitochondria have an individual K metabolism and certain apparently specific mechanisms have been elicited. The observations on the effects of DNP at high concentrations suggest that complexes may be formed between mitochondria, phosphorylated derivatives and K; breakdown and resynthesis of such complexes could be responsible for the observed exchange of mitochondrial K. If such a mechanism occurs in the intact cell it could conceivably be utilized for the function of K accumulation; these mitochondrial mechanisms may constitute an "ion-complex carrier" for K.

Summary. Rabbit liver mitochondria contain K which cannot be removed by repeated washings with NaCl solutions at 2°C. This K fraction exchanges with ambient K under appropriate metabolic conditions, indicating a dependence of K metabolism on aerobic oxidation. Orthophosphate depresses the mitochondrial K level. A newly observed polyphasic action of DNP indicates a specific relationship between mitochondrial ester phosphate and K exchange.

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Determination of Deuterium Exchange Rates Between Maternal Circulation and Amniotic Fluid.* (20217)

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The function of the amniotic fluid is clearly defined but its origin, rate of formation and its fate represent controversial subjects among investigators in this field. Morphologic studies on cellular structures involved did not supply an adequate body of information to explain the formation of large amounts of amniotic fluid in certain pathologic states nor could it explain the rapid rate of replacement in the normal pregnant organism as demonstrated by Flexner and his associates(1). These authors reached the conclusion that the amniotic fluid is completely replaced once every 2.9 hours. Such a rapid turnover rate is contrary to the prevailing opinion that the amniotic fluid represents a stagnant pool or consists of fetal urine which is swallowed by the baby. Since it would be difficult to present an acceptable technical, mechanical or morphologic explanation for the phenomenon described by Flexner, a critical examination of these experiments is in order. As a working hypothesis, one may regard the pregnant organism as a multi-compartment system which should lend itself to a mathematical approach provided certain reservations are kept in mind. Flexner and associates considered this approach but apparently discarded it in favor of approximations which are not entirely above criticism. The theoretically correct treatment of multicompartment sys-

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tems of the type described requires the exponential approach which has been investigated theoretically(2) and practically(3). It is known that labelling of the water in maternal plasma results in a gradual rise of the tracer in amniotic fluid and, conversely, one may assume that labelling the water of amniotic fluid eventually will result in an increase of tracer in the water of maternal plasma. For the purpose of the present treatment the desired information can be obtained by simplification ("lumping") to a two compartment system consisting of (1) the amniotic fluid and (2) the total body water of mother and fetus. Such a two compartment system should lend itself to the proper treatment in order to calculate the exchange (transfer) rates, a calculation which would be based on a series of determinations throughout the whole experimental period, and not on a single initial reading, as in the experiments of Flexner.

In order to determine the exchange rates or transfer rates of a compound or element in a 2-compartment system, 2 isotopes are theoretically necessary, if the rates in both directions are to be determined simultaneously. An alternate approach would be the application of one isotope in 2 different experiments where the isotope is added first to Compartment 1 and then, in a second experiment, added to Compartment 2. Since we had only one isotope of hydrogen available, the second approach was used. The treatment of multicompartment systems as outlined by Sheppard and Householder, can be used to calculate the transfer rates. The assumptions necessary for the application of Sheppard's equations are essentially those of any application of tracer experiments to biologic problems(2,4,5) and, in addition, it is assumed that deuterium is exchanged at equal and opposite rates. For this specific case the integrated equations are:

$$\frac{a_1}{a_{1(0)}} = \frac{S_1 + S_2 e^{-\rho t(1/S_1 + 1/S_2)}}{S_1 + S_2}$$

$$\frac{a_2}{a_{1(0)}} = \frac{S_1\left(1 - e^{-\rho t(1/S_1 + 1/S_2)}\right)}{S_1 + S_2} \qquad (1)$$

where a_1 and a_2 refer to the deuterium oxide concentrations in compartment 1 and 2 at time t, $a_{1(0)}$ the initial deuterium oxide concentration in compartment 1, ρ the exchange rate and, S_1 and S_2 the quantity of exchangeable species in compartment 1 and 2. Subtracting these equations leads to the expression

$$\mathrm{Log}_e\left[\frac{a - a_2}{a_{1(0)}}\right] = \rho t(1/S_1 + 1/S_2) = \frac{0.69\,t}{T_{1/2}} \qquad (2)$$

A knowledge of the equilibrium D_2O concentration in maternal blood or amniotic fluid can be used to determine the total water *i.e.*, the sum of S_1 and S_2. The volume of the amniotic fluid can be determined by direct measurement after delivery or by extrapolation of the function

$$\mathrm{Log}_e\left[\frac{(D_2O)_t}{(D_2O)_{eq.}} - 1\right] = k\,t \qquad (3)$$

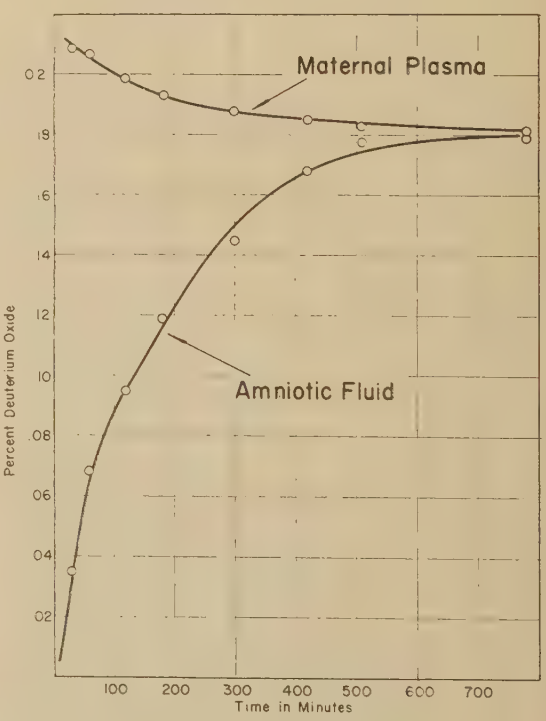

FIG. 1. Change in deuterium oxide concentration in maternal plasma and amniotic fluid following intravenous administration of 60 cc of deuterium oxide.

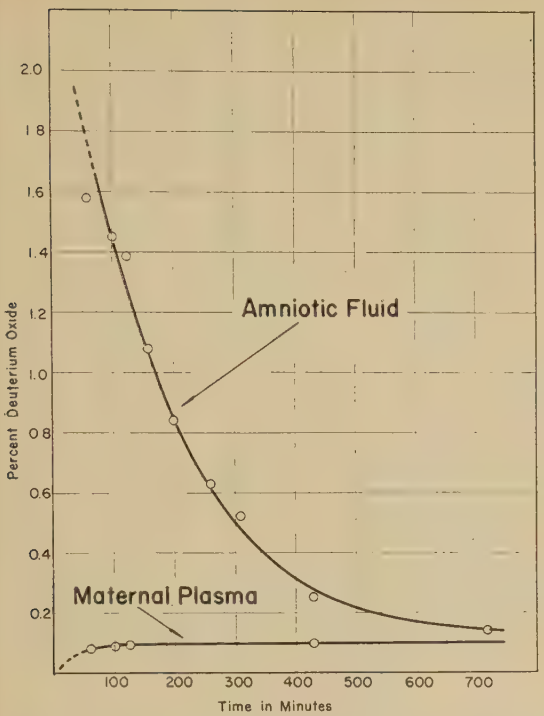

FIG. 2. Change in deuterium oxide concentration in maternal plasma and amniotic fluid following administration of 40 cc of deuterium oxide into the amniotic sac.

to zero time(5). If the assumption of equal and opposite rates is correct, substitution of the appropriate values in equation (2) should give a straight line when the values $a_1 - a_2/a_1(0)$ are plotted against time on a semi-logarithmic scale. The slope of this line then determines the half value time ($T_{½}$) and therefore the exchange rate ρ.

Experimental. (1) In the first of these experiments a normal ante-partum patient at term received 60 cc of deuterium oxide intravenously several hours before an elective Caesarean section. A sterile polyethylene catheter was introduced into the amniotic sac after transabdominal puncture by means of a Huber tip needle. Samples of amniotic fluid and maternal venous blood were then collected at pre-determined intervals and the concentration of the tracer determined by methods previously described(6,7). The results of this experiment are reproduced graphically in Fig. 1.

(2) In order to determine the transfer rate from amniotic fluid to maternal and fetal systems, the conditions of the experiment were essentially the same except that here the tracer was placed into the amniotic sac rather than the maternal system. Because of the smaller volume of the primary compartment in this experiment, slightly less deuterium oxide was used. The results of this experiment are given in graphic form in Fig. 2.

Both experiments terminated at the time of delivery by Caesarean section. A sample of cord blood, obtained just before clamping the cord, was analyzed for its deuterium oxide content. In both experiments the concentration of tracer in cord blood was found to be the same as that of the maternal plasma.

The half value time was then calculated from a plot of the function $a_1 - a_2/a_{1(0)}$ against time (Fig. 3). Substitution of the appropriate values for S_1 and S_2 in equation (2) was then used to calculate the exchange rate ρ, as 9.71 cc/min for the first experiment and 10.8 cc/min for the second. This corresponds to an exchange rate of 582 cc/hr and 648 cc/hr respectively. These values are in close agreement with, though somewhat higher

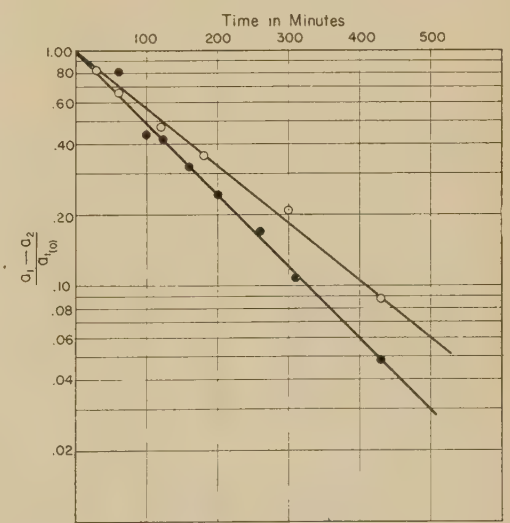

FIG. 3. Semi-log. plot of the function $a_1 - a_2/a_{1(0)}$ against time. Open circles correspond to values recorded in Fig. 1 and solid circles to those of Fig. 2. Index figure "1" refers to the primary compartment *i.e.*, the compartment which originally contained all the tracer while index figure "2" is intended to designate the secondary compartment, originally containing none of the tracer. In the 2 experiments, the order is appropriately reversed.

than, the estimations of Flexner and his associates.

Summary and conclusions. 1. Regarding the pregnant organism as a multi-compartment system the exchange rate of the water in amniotic fluid with the water of the maternal and fetal systems, was determined. By the application of trans-abdominal catheters, placed into the amniotic sac of pregnant women at term, amniotic fluid could be withdrawn at desired intervals without disturbing the continuity of the system. The mechanism of tracer (deuterium) exchange was demonstrated to be that demanded by theory, and the application of well known theoretic equations to the data so obtained permitted the calculation of absolute exchange rates. 2. In 2 experiments where the isotopic tracer was first placed in one compartment (mother) and then in the other (amniotic fluid) the exchange rates in both directions were found to be about 600 cc per hour. The observations of Flexner and his associates on the exchange rate of the water of the amniotic fluid are thus confirmed in principle.

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Effects of Sex Hormones on Serum Lipoproteins in Rabbits.* (20218)

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Ultracentrifugal analyses of blood serum in human beings have indicated differences in the lipoprotein pattern between male and female: beta-lipoprotein concentrations are approximately 25% smaller in women than men (1) and, "The incidence of measurable concentrations of the Sf 10-20 class is significantly higher in males from 20 to 40 years of age than in females of the same age group"(2). In similar investigations, Barr, *et al.*(3) using alcohol fractionation technic, found that administration of estrogens to atherosclerotic men resulted in an increase in alpha and a decrease in beta-lipoproteins. However, Glass, *et al.*(4), did not observe any change in the serum lipids or low density lipoproteins (Sf 12-20) following treatment with estradiol of 55-65-year-old male and female patients.

There are thus indications that androgens and estrogens may have opposite effects on lipoproteins and, therefore, be important in the regulation of lipoprotein concentration. Since androgens have not been studied, the present investigation was undertaken in rabbits subjected to alternate treatment with stilbestrol and testosterone.

Methods. Nine sexually immature rabbits (5 males and 4 females) of mixed strain with an average body weight of 1.35 Kg were kept without treatment during a preliminary period of 15 days, at the end of which they were castrated. During the following 5 months they were treated with stilbestrol or testosterone; each period of treatment was followed by a control period in order to permit a complete resorption of the drug. Diethylstilbestrol in oil solution was injected subcutaneously at the daily dose of 1 mg; testosterone in aqueous crystalline suspension was injected at the

* This investigation was supported by a grant from the National Heart Institute, National Institutes of Health, Bethesda, Md.

TABLE I. Effects of Diethylstilbestrol and Testosterone on Serum Lipoproteins and Cholesterol.

Date	Treatment	$-S_{1.21}$ in mg/100 ml serum	>70	40–70	20–40 (35)	1–10 (7)	Serum cholesterol in mg/100 ml
5/9	—				50 (12–128)	69 (12–95)	41 (27–65)
5/22	Castration				78 (32–95)	96 (76–104)	66 (44–101)
6/24–7/7	Diethylstilbestrol, 1 mg/day				37 (21–80)	97 (57–142)	47 (36–64)
7/15–7/30	Testosterone, 20 mg/day		16 (0–66)	7 (0–21)	56 (16–62)	122 (104–130)	50 (39–64)
8/25	—				80 (17–170)	103 (71–127)	62 (38–71)
9/12	—				81 (49–141)	102 (47–164)	70 (48–114)
9/26–10/8	Testosterone, 20 mg/day		20 (0–59)	16 (0–35)	77 (30–138)	97 (54–115)	71 (54–91)

dose of 20 mg. The animals were fed on commercial rabbit pellets and had tap water to drink. As indicated in Table I, blood analyses were performed at intervals in the course of the experiment. Twenty ml of blood were drawn from the femoral artery and the ultracentrifugal serum lipoprotein pattern determined at a density of 1.21 as described by Green, Lewis and Page(5). On the same sample, total serum cholesterol was estimated by the method of Kendall *et al.*(6).

Results. The normal lipoprotein pattern of immature rabbits' sera showed 2 main peaks; a less dense component (beta-lipoprotein) with a negative sedimentation of -S 35 and a more dense component (alpha$_1$-lipoprotein) –S 7 (Table I). The concentration of the lipoproteins and cholesterol showed great individual variations, probably due to the mixed strain of the animals; –S 35 averaged 50 mg (range 12 to 128 mg per 100 ml); –S 7 averaged 69 mg (range 12 to 95 mg per 100 ml); cholesterol averaged 41 mg (range 27 to 65 mg per 100 ml).

Following castration, there was an increase in the concentration of –S 35 and –S 7 components as well as in cholesterol; this increase averaged respectively of 28, 27, and 25 mg per 100 ml. After treatment with diethylstilbestrol, the –S 35 lipoprotein and cholesterol concentrations decreased to values near the precastration levels, while the –S 7 remained unchanged. No abnormal components were found as a result of castration or of estrogen treatment.

Following testosterone injections, the lipoprotein pattern was markedly altered by the presence of –S (40-70) and –S >70 components, which are not usually present in the serum of normal or castrate rabbits. These changes are significant since they were present during the 2 periods of treatment and disappeared when testosterone treatment was discontinued.

This effect of testosterone on lipoproteins is further demonstrated in one rabbit, which is not included in the table because of abnormal components (–S > 70 and –S (40-70)) present during the control period: the respective concentrations of these 2 components were 36 and 12 mg per 100 ml. Following castration, these 2 abnormal components disappeared, but reappeared during testosterone treatment to reach the following values: –S > 70, 142 mg and –S (40-70) 33 mg per 100 ml, which are higher than the precastration levels. On substitution of testosterone by stilbestrol injections, these components again disappeared.

Discussion. Glass *et al.* observed no effects in human beings on "low density" lipoproteins as determined by ultracentrifugation at a density of 1.06 from physiologic or pharmacologic doses of estradiol(4). This low density fraction, which was present only in one of our rabbits at the beginning of the

treatment, disappeared under the influence of estrogen. In the other rabbits the only effect noted was a decrease in the concentration of the –S 35 fraction. The absence of a concomitant increase in alpha$_1$-lipoproteins in rabbits contrasts with the results of Barr *et al.* (3), obtained by alcohol fractionation and confirmed by ultracentrifugation at a density of 1.21(7), in patients receiving estrogens. Nava and Zilli(8) also observed an increased concentration of serum alpha-globulin following treatment of normal healthy men and women with estradiol or diethylstilbestrol. This variation in response to estrogens is associated with difference of species and difference in lipoprotein pattern of rabbit and human serum. The most significant finding in the present experiments is the increase in the low density lipoproteins (–S (40-70) and –S > 70) following treatment with testosterone; it explains the sex difference and may offer a partial explanation for the greater susceptibility of male than female to atherosclerosis.

Summary. The serum lipoprotein patterns of immature rabbits were determined by ultracentrifugation at a density of 1.21. Two main peaks were resolved: one at –S 35 (20-40) and another at –S 7 (1-10). After castration, these animals were treated with diethylstilbestrol and with testosterone. Only testosterone altered significantly and regularly the lipoprotein pattern by the appearance of two abnormal components; one, –S > 70 and the second, –S (40-70); both disappeared on cessation of treatment.

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Observations on Preservation of Human Spermatozoa at Low Temperatures. (20219)

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The survival of human spermatozoa, after freezing at low temperatures, is not a new observation. Mantegazza(1), as recorded by Davenport(2), was perhaps the first to report this phenomenon. Jahnel(3), Shettles(4), and Hoagland and Pincus(5) have since shown, by various technics, that human spermatozoa will survive temperatures of –79°C to –269.5°C. In an attempt to evaluate the merits of rapid freezing and to reconcile the varied results of these reports, Parkes(6) showed that survival is far better when 1.0 cc of semen is frozen in ampoules rather than tiny amounts in fine capillary tubes. He states it was fairly certain that, under optimum conditions, a large number of human spermatozoa could survive prolonged freezing, but expressed considerable doubt that the speed of freezing and thawing was the primary factor

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in their survival. However, his observation of increased survival with increased volume gave us evidence for the importance of slow freezing, as greater volume extends the freezing time. Further indication of this trend was given in the data of Hoagland and Pincus(5), where greatest survival resulted through the use of foamed semen produced by air bubbles which certainly insulated much of the semen and therefore prolonged its freezing time. Moreover, Luyet(7) noted that the air cushion formed about an object immersed in liquid nitrogen at –196°C (the apparently preferred freezing medium) made it a method of freezing 5 times slower than isopentane at –150°C and even slower than a dry ice bath at –77°C. A comparison of different freezing methods seemed logical and, since glycerol had been shown to increase the resistance of human spermatozoa to freezing, Polge, Smith, and Parkes(8), it was decided to employ this protective substance in this study.

The purpose of this communication is to present some findings concerning the conditions necessary for the maximum survival of human spermatozoa after freezing and storage at low temperatures.

Methods. Collection of semen. Semen was obtained on 3 occasions from each of 5 different donors. Collections were made after 5 days of abstinence to minimize production variation and to coincide with the spermatozoal production peak. These semen specimens were considered normal by the usual seminological tests, Farris(9). *Pretreatment of semen.* Preliminary tests indicated that 1 part of absolute glycerol added to 9 parts of liquefied semen, maintained semen quality with a minimum dilution. The glycerol was added immediately after liquefaction and, depending upon the volume of semen, 0.5 ml or 1.0 ml portions of the treated semen were placed in 12 mm x 75 mm bacteriological test tubes. The tubes were then corked and allowed to stand for 30 minutes at room temperature prior to freezing. *Freezing of semen.* In the probable order of fastest to slowest freezing methods, isopentane (IP) cooled to –150°C by liquid nitrogen, dry ice in acetone slush (DIA) at –79°C, liquid nitrogen (LN) at –196°C, and dry ice in an insulated box (DI) at –70°C were compared as refrigerants. Luyet's(7) and our own gross observations gave evidence that this is the actual order of freezing time. Since initial experiments had shown that glycerol increased survival in all 4 freezing methods, only glycerol treated semen was employed. Each sample was distributed in 4 tubes. The first three tubes were immersed for 5 minutes in their respective baths of IP, DIA, and LN. These 3, along with the fourth, were then placed in DI for periods of one to 3 months, at which time they were quickly thawed in a 37°C water bath. *Tests of semen quality.* In previous observations, objective measurements of spermatozoal survival were either lacking or limited to motility counts. It was decided to make use of additional valid criteria of measurement, both before freezing and after thawing. The usual blood hemacytometer method was employed to compute the per cent motility while the eosin-nigrosin staining technic of Blom(10) provided information concerning viability and morphology. The speed and type of movements were noted as well as the length of time spermatozoa were motile at room temperature.

TABLE I. Average % Survival in 15 Series of Glycerol Treated Human Spermatozoa after Freezing by Methods Indicated, then Stored in DI, 1 to 3 Months and Quickly Thawed in a 37°C Water Bath. % viability based upon eosin-nigrosin test.

Speed of freeze	Method of freeze	% viability Before freeze	% viability After thaw	% survival
Fastest	IP	64	15	23
Fast	DIA	64	27	42
Slow	LN	64	9	14
Slowest	DI	64	43	67

Results. 1) The results of our study are summarized in Table I. The greatest per cent survival was obtained in specimens which were frozen and stored in dry ice (DI). The results obtained from 15 semen samples which were frozen in dry ice (DI) are summarized in Table II.

2) The per cent survival, as measured by the eosin-nigrosin method, was usually identical (within 2%) with the per cent motility as computed by hemacytometer counts. This agrees with the findings of Williams and Pollak(11).

3) Several semen samples were examined

TABLE II. Breakdown of Those Data in Table I Which Concerns DI as a Freezing Method.

Donor	Sample	% viability Before freeze	% viability After thaw	% survival
A	1	73	50	69
	2	68	50	74
	3	69	52	75
B	1	50	34	68
	2	61	39	64
	3	53	37	70
C	1	65	49	76
	2	63	48	76
	3	69	53	77
D	1	67	42	62
	2	68	39	58
	3	61	19	31
E	1	68	46	68
	2	62	43	70
	3	60	41	69
Avg		64	43	67

after storage for one, 2, and 3 months, and no loss in per cent survival was noted.

4) No significant difference was observed between the results with 0.5 cc and 1.0 cc of semen.

5) There was no apparent difference between the type, speed, and duration of motility of glycerol treated spermatozoa before and after preservation. No morphologic alterations were obvious in our observations.

Discussion. If the order of freezing speeds assumed for the refrigerants employed in this study is correct, the results obtained with liquid nitrogen (LN) are not consistent with the trend toward increased survival with slower methods of freezing (Table I). This problem, as well as the one concerning the protective action of glycerol, is now being attacked experimentally by us.

There is evidence under our experimental conditions that, except for one sample from donor D (Table II), the per cent survival is fairly constant in semen samples from the same donor. There seems to be some variation, though, from donor to donor. Shettles (4) maintains there is a marked individual variation in the sensitivity of human spermatozoa to extremely low temperatures. We feel, however, that a greater number of observations are necessary before any definite statements can be made on either score.

Clinical application of practical storage banks for human spermatozoa in infertility problems is now in progress. Artificial inseminations to test the ability of frozen human spermatozoa to fertilize and induce normal embryonic development are underway in this laboratory.

Summary. Initial experiments indicate that when 9 parts of liquefied human semen are treated with 1 part of absolute glycerol and placed in an insulated box containing dry ice, it can be stored for at least 3 months and, after quick thawing in a 37°C water bath, it will show an average spermatozoal survival of 67%, a figure superior to those realized with faster freezing methods. Such revived spermatozoa show no detectable alterations in motility or morphology.

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Effect of Histoplasmin Skin Testing on Serologic Results.* (20220)

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The booster effect of repeated positive histoplasmin skin reactions on the collodion agglutination test for histoplasmosis has been reported previously(1). The purpose of this study was to compare the effect of skin testing upon the histoplasma complement-fixation tests using both yeast phase and histoplasmin antigens, upon the collodion agglutination test, and upon the blastomyces and coccidiodes complement-fixation tests.

Methods. Twenty-five healthy volunteer medical students received 6 weekly intradermal injections with 0.1 ml of lot H-42 histoplasmin (1:100).† Blood for serum antibody studies was obtained prior to each skin test and at 7,26,30 and 40-44 weeks after the first skin test, whenever possible or indicated. Seven additional volunteers were bled prior to and weekly for 5 weeks after a single positive skin test. Histoplasmin lots H-15‡ H-17† and H-37† were used in parallel in the collodion agglutination test(2,3). Lot H-15 histoplasmin, lot 47-54§ coccidioidin and ground yeast phase histoplasma and blastomyces antigens were employed in 50% end-point complement-fixation tests(4,5).

Results. Fourteen of the 25 volunteers were skin-test positive. Of this number 12 developed antibodies detectable by one or more of the serologic tests for histoplasmosis after 2 or more skin tests. None of the 11 skin-test negative reactors developed demonstrable antibodies with any of the serologic procedures after 6 weekly skin tests. Similarly, none of 7 showed any humoral antibodies for 5 weeks after a single positive skin test.

Collodion agglutination. Eight of 14 (57.1%) skin-test positive persons developed 4+ agglutinations in peak titers varying from 1:10 to 1:40. As summarized in Table I, the sera of 4 (Nos. 5,10,12,13) became positive after 2 skin tests, the other 4 (Nos. 1, 3, 6, 14) after 3 skin tests, respectively. At the 5th and 6th weeks the serum of one (No. 12) became negative. Positive agglutinations were observed in only 2 instances (Nos. 5 and 14) 21 weeks after the last skin test, but these were negative when checked at 40 weeks. Thus the positive collodion agglutination reactions occurring after repeated positive skin tests were relatively transient in nature, and all were negative 35 weeks after the last skin test. This is in accord with previous studies (1).

Histoplasmin complement-fixation. When histoplasmin was used as antigen, 12 of 14 (85.7%) skin-test positive persons developed complement-fixing antibodies in titers varying from 1:5 to 1:40. Four were positive after the 2nd skin test, 8 after 3, 11 after 5, and the 12th person at 26 weeks (See Table I). At this latter time antibodies were still present in the sera of 8 of the 9 previously positive, while at 30 weeks, 4 of 5 specimens, previously positive at 26 weeks, fixed complement. At 40 to 44 weeks, 3 (Nos. 2, 7, 10) still remained positive. For comparative purposes 8 serum specimens from Nos. 2,7,10 and 11 taken at 26 to 44 weeks were sent to the Communicable Disease Center at Chamblee, Georgia‖ where histoplasmin is used as the antigen in complement-fixation. Their results confirmed our findings in all instances. In contrast to the transient nature of positive reactions in the agglutination test or in yeast-phase antigen complement-fixation (*vide infra*), the duration of positive reactions was much longer with histoplasmin as antigen in

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‡ Supplied by Dr. Arthur Hillegas, Parke Davis & Co.

§ Supplied by Dr. Charles E. Smith, University of California.

‖ Test performed by Dr. Joseph Schubert.

TABLE I. Effect of Repeated Positive Histoplasmin Skin Tests on Serologic Responses.†

Subject	Test	Time (wk) ‡	1*	2*	3*	4*	5*	6	26	30	40-44
1.	CA	0	0	10	20	10	10	0			0
	YF	0	5	5	5	5	10	0			0
	H	0	5	10	10	10	10	10			0
	B	0	0	0	5	5	5	0			0
2.	YF	0	20	10	20	20	20	0	0		0
	H	0	10	10	20	10	10	10	5		5
	B	0	10	10	20	20	20	0	0		0
3.	CA	0	0	10	10	10	10		0		0
	YF	0	0	0	0	5	5				0
	H	0	0	0	0	5	10				0
5.	CA	0	20	40	40	40	40	10			0
	YF	0	0	0	0	0	10	0			0
	H	0	0	10	10	10	20	5			0
6.	CA	0	0	5	10	10	5	0			
	YF	0	10	10	5	10	20	0			
	H	0	0	5	0	0	0	0			
	B	0	10	10	5	5	10	0			
7.	YF	0	0	5	10	20	20	0	0		0
	H	0	0	5	20	20	10	10	10		5
	B	0	5	5	20	20	20	0	0		0
8.	YF	0	0	0	0	5	5	0			
	H	0	0	0	0	5	5	0			
	B	0	0	0	0	5	10	0			
10.	CA	0	20	40	40	40	40	0	0		0
	YF	0	10	10	20	20	20	0	0		0
	H	0	5	40	40	20	40	10	10		10
	B	0	5	10	10	10	20	0	0		0
11.	YF	5	5	5	10	10	10	0	0		0
	H	0	5	5	10	10	10	5	5		0
	B	10	10	10	20	20	20	0	0		0
12.	CA	0	40	40	10	0	0	0	0		
	YF	0	ND	ND	5	ND	ND	0	0		
	H	0	ND	ND	5	ND	ND	5	0		
13.	CA	0	10	10	10	20	10	0			0
	H	0	0	0	0	0	0	5			0
14.	CA	0	0	20	40	40	40	10			0
	YF	0	10	10	40	40	40	0			0
	H	0	0	5	10	10	10	0			0
	B	0	5	20	40	40	40	0			0

* Positive histoplasmin skin test.

† Only serologic tests developing positivity are listed.

‡ 0 wk all 0.

CA, collodion agglutination; YF, yeast phase histoplasma complement fixation; H, histoplasmin complement fixation; B, blastomyces yeast phase complement fixation; ND, not done.

complement-fixation tests.

Histoplasma yeast phase complement-fixation. Eleven of the 14 (78.6%) skin test positive persons demonstrated complement-fixing antibodies in titers ranging from 1:5 to 1:40 between the 2nd and 6th weeks after the initial skin test, *i.e.* one week after the 2nd to 5th weekly skin test. None of these persons' sera was positive when samples were obtained at 26 weeks. Thus, with yeast phase antigen a relatively transient rise in antibodies usually followed skin testing and no positive sera were detected 21 weeks after the last skin test (See Table I).

Blastomyces yeast phase complement-fixation. Cross-reacting titers were observed in the sera of 8 of 14, or 57.1%. These appeared at similar time intervals as with homologous antigens and frequently in equal or occasionally even higher titer. However, as with yeast phase histoplasma antigens, all sera were negative when checked at 26 weeks, or 21 weeks after the last skin test (Table I).

Coccidioides complement-fixation. None of the sera reacted with coccidiodin antigen following histoplasmin skin testing.

Discussion. With the increased use of histoplasmin as a skin testing agent one must consider its potential effect on homologous and heterologous serologic tests. It was, therefore, not surprising that antibodies were demonstrated frequently following 2 or more positive skin tests. When the same type of antigen, histoplasmin, was used in complement-fixation as was used in skin testing, the antibody titer demonstrated more permanence than when yeast phase antigen was used. Since no serologic alterations were observed in skin test negative individuals, one could assume that in the positive skin reactors a previously attained immune response was stimulated by skin testing. The fact that such reactions were found in healthy individuals would suggest that a similar or an even greater response could be obtained during the active or convalescent stages of histoplasmosis. In our experience, peak titers with yeast phase complement-fixation tests in both animal and human infections have fallen by 5-8 months and low or negative results are seen after 12 to 18 months(6,7). Other studies in which histoplasmin was employed as antigen have shown positive tests as late as 3-4 years after the onset of the disease when the patient had been clinically well for 1-2 years(8). However, a review of these latter cases reveals numerous repeated positive skin tests. Positive agglutination reactions did not persist as long as did complement-fixing antibodies although histoplasmin was used as antigen

in both tests. However, the latter test is more sensitive when antibodies are low in titer and the collodion agglutination test is frequently negative after the 3rd month or so of active disease(7,9). Cross-reacting tests with blastomyces antigen were not unexpected, and have been described previously(2,4,5). Results from these laboratories demonstrated that at different stages of histoplasma infections heterologous blastomyces antibody titers often equaled and sometimes surpassed, the homologous histoplasma titers(10).

It is felt that repeated skin tests would alter and confuse the utilization of serologic data in the diagnosis of histoplasmosis.

Conclusions. Repeated positive histoplasmin skin tests in normal medical students caused false positive reactions in the collodion agglutination test (57.1%) and in the complement-fixation test with histoplasma yeast phase antigen (78.6%) or with histoplasmin as antigen (85.7%). The response was relatively transient in the former 2 tests, and negative titers were usually restored by 21 weeks after the last skin test. However, positive results were noted as late as 21, 25 and 39 weeks in complement-fixation tests employing histoplasmin as antigen. A transient response was observed in complement-fixation studies with cross-reacting yeast phase blastomyces antigens (57.1%), but no sera reacted in the same test with coccidiodin antigen. A single positive skin test and repeated negative skin tests did not alter serologic results. Thus, repeated histoplasmin skin testing of skin-test positive persons may invalidate complement-fixation data for long periods with histoplasmin as antigen, and for much shorter periods with yeast phase histoplasma and blastomyces complement-fixation or histoplasmin collodion agglutination tests.

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Effects of Large Doses of Desoxycorticosterone Acetate, Cortisone Acetate and ACTH in Intact Rhesus Monkeys.* (20221)

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In the course of a study designed primarily to investigate the effects of relatively large doses of desoxycorticosterone acetate, cortisone acetate and ACTH on the morphology of the adrenal cortex of the rhesus monkey(1), additional observations were recorded relating to the physiological effects of these hormones in the intact animal. These are reported in the present paper.

Materials and methods. The 14 monkeys (9 males and 5 females) used in this study were quartered in individual cages located in an air-conditioned room maintained between 78° and 80°F. Food(2) and water were supplied *ad libitum* except for a 12-hour fast preceding each bleeding. The monkeys were bled at three- to four-day intervals prior to and

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TABLE I. Effects of ACTH, Cortisone Acetate and Desoxycorticosterone Acetate (DCA) on Adrenal and Body Weights of Intact Rhesus Monkeys.

Animal No.	Sex	Treatment	Dose/day, mg	No. days	Initial body wt, g	Final body wt, g	Adrenal wt, mg†
28	♂	ACTH (Wilson), intramusc. inj.	80*	6	5870	6055	1022
32	♂	"	80*	8	4180	4400	1148
39	♂	"	80*	8	4310	4050	1187
14	♂	ACTH (Astwood) in gelatin, subcut. inj.	.5‡	8	6445	6605	774
89	♀	"	5.0	34	2800	2950	486
66	♀	"	1.0	34	2800	2915	919
42	♂	DCA (aqu. suspension), intramusc. inj.	25	42	4880	4460	433
43	♂	"	25	42	4225	3810	321
45	♂	Cortisone (aqu. susp.), intramusc. inj.	50	42	4125	3390	145
40	♂	"	50	14	4085	4015	295
8	♂	"	25	38	2433		
			50	30		2380	108
E1	♀	"	50	33	3815	3965	148
E2	♀	"	50	33	3700	3650	170
E3	♀	"	50	33	3820	3650	209

* Administered in 4 daily inj. of 20 mg each.
† Wt of one gland. Range of adrenal wt for 15 normal animals of simliar body wt is 216-437 g.
‡ 80 U.S.P. units/mg(8).

following the initiation of treatment. The blood samples were analyzed for glucose, nonprotein nitrogen, amino acid nitrogen, inorganic phosphorus, sodium, potassium and chloride(2). The hematocrit was also determined. Body weights were recorded at the time of bleeding. The various treatments are summarized in Table I. Glucose tolerance tests were performed in 3 monkeys (E1, E2 and E3) by intravenous infusion of glucose (75 mg per kg body weight). At the termination of the treatment periods the animals were adrenalectomized(2) and the recovered adrenal glands were weighed and examined histologically.

Results. 1) *ACTH.* The administration of this substance resulted in enlargement of the adrenal glands (Table I) and stimulation of the adrenal cortex in terms of histological and histochemical criteria(1). Aside from these responses, ACTH, as administered in this study, failed to effect significant changes in hematocrit and in the various blood constituents studied (Fig. 1). No loss in body weight was recorded for any animals receiving ACTH (Table I).

2) *Desoxycorticosterone acetate.* This treatment effected a loss in body weight (animals 42 and 43) over the period of hormone administration (Table I). A reduction in food intake was noted during this period. No significant changes in adrenal weights were observed (Table I). The blood levels of inorganic phosphorous and potassium were lowered slightly, but significantly, during the course of hormone administration. The hematocrit values gave evidence of moderate

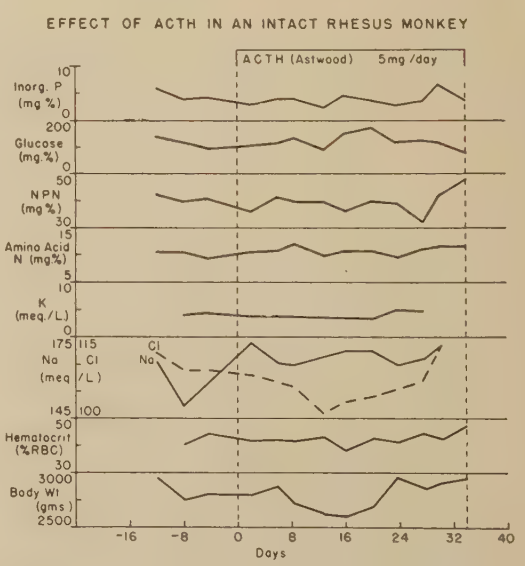

FIG. 1. Effects of ACTH (Astwood—5 mg [400 I.U.] per day) in monkey 89.

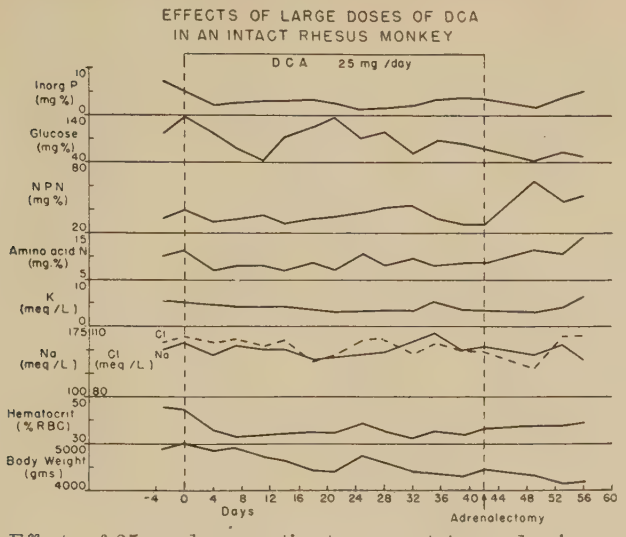

FIG. 2. Effects of 25 mg desoxycorticosterone acetate per day in monkey 42.

hemodilution. No significant changes were observed in the concentrations of amino acid nitrogen, non-protein nitrogen, glucose, sodium and chlorides (Fig. 2). Approximately 10 days after the initiation of DCA administration, both animals exhibited a marked polydipsia which was accompanied by severe edema of the scrotal and penile regions. These manifestations lasted for a few days, subsided and did not recur during the remainder of the treatment period.

3. *Cortisone acetate.* Of the 6 animals given cortisone, only one showed a marked loss in body weight (No. 45, Table I). This monkey became anorexic and listless as the experimental period progressed and a considerable reduction in the muscle mass of the lower extremities was noted. The influence of cortisone on the blood constituents as recorded for monkey no. 45 are shown in Fig. 3. The only salient changes found in this and the other animals with regard to the blood picture were a decrease in the serum potassium concentrations and a lowering of the hematocrit. It is noteworthy that hyperglycemia was not observed at any time in the course of cortisone administration. Cortisone (50 mg per day for 30 days) similarly was without effect on glucose tolerance in the 3 monkeys so studied (Fig. 4). Adrenal weights were markedly decreased in all monkeys receiving the larger doses of cortisone (Table I).

Discussion. That ACTH in very large doses (to 400 mg per day) can be ineffective in producing significant increases in blood sugar has been demonstrated in the cat(3) and in the mongrel dog(4). It appears from the present data that the rhesus monkey must be grouped with these species rather than with man, where as little as 12.5-50 mg of ACTH (Li's ACTH protein) will increase fasting blood sugar levels(5). Raben *et al.*(8), using

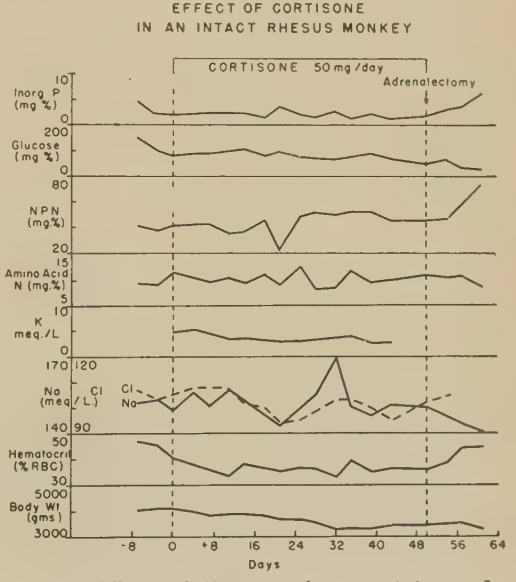

FIG. 3. Effects of 50 mg cortisone acetate per day in monkey 45.

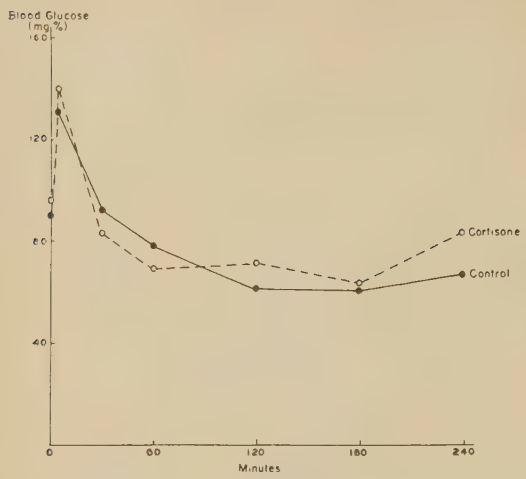

FIG. 4. Glucose tolerance curves before and after treatment with 50 mg cortisone acetate per day for 30 days. Each curve represents mean values of animals E1, E2 and E3 before and after hormone administration.

the Astwood corticotropin, observed symptoms of overdosage in patients receiving 0.19 to 0.3 mg per day whereas no such effects were observed in a monkey (No. 89) which received 5 mg of the same preparation per day for 34 days.

Cortisone likewise failed to induce hyperglycemia or to alter glucose tolerance although an electrolyte effect, *viz.* a depression in serum potassium, was observed. This finding is in agreement with the recent report of Sirek and Best(6) that in dogs, large doses of cortisone evoke a diabetes insipidus-like response but do not elevate the levels of blood sugar or change the glucose tolerance curves. Such effects have been associated with the action of large doses of desoxycorticosterone as shown in the dog by Kuhlman *et al.*(7) and as demonstrated in the present study where a transient polydipsia and a lowering of serum potassium was obtained in monkeys given desoxycorticosterone. It is of interest that this decreased potassium concentration was not accompanied by corresponding increases in serum sodium and chloride levels.

The observation that only one of the monkeys receiving cortisone exhibited an important loss in body weight cannot be explained at present, but it is noteworthy that this loss was not reflected in changes of blood amino acid or non-protein nitrogen concentrations.

Summary. The effects of large doses of ACTH, cortisone acetate and desoxycorticosterone acetate were investigated in the intact rhesus monkey. ACTH was without effect on hematocrit and the blood levels of glucose, non-protein nitrogen, amino acid nitrogen, inorganic phosphorous, sodium, potassium and chloride, although the adrenal glands were strongly stimulated as evidenced by adrenal gland weight and histological examination. Cortisone acetate and desoxycorticosterone acetate effected a decrease in serum potassium and a lowering of the hematocrit without altering the levels of the other blood constituents. One of the 6 animals receiving cortisone suffered a significant weight loss, but hyperglycemia and elevated glucose tolerance curves were never observed. A transient polydipsia and edema were noted in the course of desoxycorticosterone administration.

We wish to thank Dr. E. B. Astwood for generous supplies of his corticotropin preparations, Dr. P. L. Perlman of the Schering Corp. for the desoxycorticosterone acetate and Dr. E. Alpert of Merck and Co. for the cortisone acetate. The suspensions of DCA were kindly prepared by Drs. L. M. Ohmart and M. J. Stoklosa of the Massachusetts College of Pharmacy. The technical assistance of Mrs. L. W. Parker is gratefully acknowledged.

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Blood Adrenocorticotrophin in Children with Congenital Adrenal Hyperplasia.* (20222)

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The oxycellulose technic(1) for the analysis of ACTH in blood is now being applied to problems in pituitary-adrenocortical physiology. The preliminary data presented in this report suggest that the titer of ACTH is greater than normal in the blood of patients with adrenogenital syndrome.

Methods. 24 subjects were studied: 9 afebrile children without endocrine disease were included in the control group; 8 children (7 genetic females and 1 genetic male) with adrenogenital syndrome were included in a second group; and 7 children (3 with acute rheumatic fever, 2 with Sydenham's chorea, 1 with acute glomerulonephritis and 1 with idiopathic hypoglycemosis) were included in a miscellaneous group. A blood sample was obtained from either an internal jugular or an antecubital vein and added immediately to 4 volumes glacial acetic acid. The blood-glacial acetic acid mixture was processed by the oxycellulose‡ technic for the concentration of adrenocorticotrophin (ACTH)(1) and assayed in male hypophysectomized rats by the adrenal ascorbic acid-depletion method(2). In every assay, freshly prepared ACTH standard solutions were tested with blood eluates. The standard was assayed at 0.25 and 1.0 milliunit (mu§) or at 0.25, 0.5 and 1.0 mu per 100 g test rat. The eluate of a blood sample was divided so that a dose equivalent to 20, 40 or 50 ml of blood was injected into each of 2 to 5 assay rats.

Results. The individual and average responses of the test rats to given doses of blood together with the corresponding individual and average responses to the low dose of standard tested on the same day are presented in Table I.

The results are expressed in terms of adrenal ascorbic acid depletion, that is, the concentration of ascorbic acid in the left adrenal, removed prior to injection, minus the concentration of ascorbic acid in the right adrenal, removed one hour after injection of the test material. Negative values indicate that the concentration of ascorbic acid in the right adrenal of the test animal is higher than the concentration in the left adrenal. Fluctuations about zero for the difference between the concentration of ascorbic acid in the left and in the right adrenal following the injection of 0.9% sodium chloride solution represent, in part at least, the error of the analytical technics employed. Biological variation may also contribute to this fluctuation about zero. Results of an experiment in which 0.9% sodium chloride solution was injected follow: -40, 10, 7, -3, 8, 17, 12, 47, 12, 7, -13, -19, -12, 17, -18, 24, -11, -18, -15, -6, 24, 12, 2, 9, 30, -17, 9, 18, -10, average *3*.

In the control group, eluates equivalent to 20 to 50 ml of blood per test rat did not induce a significant depletion of adrenal ascorbic acid, whereas 0.25 mu ACTH per 100 g test rat induced a significant degree of depletion. Therefore, it can be stated that the concentration of ACTH in the blood of these control subjects appears to be less than 0.5 mu per 100 ml. Eluates equivalent to 40 ml blood obtained from untreated children with adrenogenital syndrome produced a significant depletion of adrenal ascorbic acid. Although the single-dose assays employed do not permit a

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‡ Samples of oxidized cellulose (10-12% COOH) were generously supplied by the Research Laboratories, Tennessee Eastman Co.

§ One mu equals one-thousandth of a U.S.P. Unit of ACTH which is defined as the activity of one mg of the International Standard ACTH (La-1-A).

TABLE I. Blood ACTH in Children.

	Patient, sex and age (yr)	Dose (ml)	Blood sample Responses* Individual	Blood sample Responses* Avg	Standard ACTH 0.25 mu Responses* Individual	Standard ACTH 0.25 mu Responses* Avg
Control subjects	K.M. ♀ 4	20	−24, −25	−25	83, 66, 38, 54, 60, 111	69
	S.B. ♀ 3	40	−5, 18	7	83, 66, 38, 54, 60, 111	69
	L.S. ♀ 7	"	6, 18	12	27, 20, 35, 92, 50	45
	J.A. ♂ 3	20	5, 22	14	83, 66, 38, 54, 60, 111	69
	G.S. ♂ 9	40	−31, −16	−24	27, 20, 35, 92, 50	45
	L.S. ♂ 12	"	24, 14	19	66, 85, 66, 134, 91	88
	L.S. ♂ 8	"	−27, −26, 38	−5	22, 29, 25, 25, 105, 102	51
	L.M. ♂ 13	"	−30, −53, 36	−16	8, 12, 74, 11, 28, 63, 77, 22	37
	D.C. ♂ 9	"	−12, 26, 2	5	76, 85, 88, 111, 85	89
Adrenogenital syndrome prior to therapy	W.C. ♀ 4	20	29, 29, 29	29	83, 66, 38, 54, 60, 111	69
	S.Z. ♀ 9	40	76, 118, 69, 45	77	83, 30, 92, 54, 66, 68, 58	64
	F.P. ♀ 10	"	89, 20, 66	58	127, 102, 59, 114	101
	N.W. ♀ 4	"	62, 46, 84, 57	62	83, 30, 92, 54, 66, 68, 58	64
	J.C. ♀ 14	"	42, 56, 27	42	76, 85, 85, 111, 85	88
Adrenogenital syndrome during cortisone therapy	W.C. ♀ 4 (40)†	"	29, 6, −7, −10	5	27, 20, 35, 92, 50	45
	F.P. ♀ 10 (10)	"	−42, −28, −8, 23	−14	8, 12, 74, 11, 28, 63, 77, 22	37
	S.B. ♀ 3 (270)	"	−52, −15, −6, 3	−18	83, 30, 92, 54, 66, 68, 58	64
	E.Wi. ♀ 2½ (37)	"	32, −2, −42	−4	93, 83, 117, 84, 78, 76, 77	87
	E.Wi. ♀ 2½ (89)	20	46, −41, −48	−14	25, 19, 35, 25, 33	27
	R.Wi. ♂ 4 (33)	40	−40, 27, −44, −6	−16	93, 83, 117, 84, 78, 76, 77	87
	R.Wi. ♂ 4 (86)	20	11, 22, 23	19	25, 19, 35, 25, 33	27
Adrenogenital syndrome after cortisone therapy	R.W. ♀ 6 (60)‡	40	56, 18, 18, −20	18	66, 85, 66, 134, 91	88
	S.B. ♀ 3 (90)	"	−47, 19, 3, 51	7	8, 12, 74, 11, 28, 63, 77, 22	37
	E.Wi. ♀ 2½ (47)	20	75, 54, 41	57	37, 10, 21, 68, 90	45
	E.Wi. ♀ 2½ (30)	"	45, 52, 45	47	51, 93, 40, 16	50
	R.Wi. ♂ 4 (47)	"	5, 39, −17	9	37, 10, 21, 68, 90	45
	R.Wi. ♂ 4 (30)	"	−3, 12, 20	10	51, 93, 40, 16	50
Miscellaneous group	J.P. ♀ 8 (acute glomerulonephritis)	40	8, 8, 26	14	8, 12, 74, 11, 28, 68, 77, 22	37
	M.B. ♀ 4 (idiopathic hypoglycemosis)	"	47, 90	69	22, 29, 25, 25, 105, 102	51
	G.S. ♂ 9 (acute rh. fever without carditis)	"	16, −3, 38	17	8, 12, 74, 11, 28, 68, 77, 22	37
	D.S. ♂ 10 (acute rh. fever with carditis)	"	137, 132, 110	126	8, 12, 74, 11, 28, 68, 77, 22	37
	J.B. ♂ 14 (acute rh. fever with carditis—prior to cortisone Rx)	"	184, 155, 97	145	66, 85, 66, 134	88
	J.B. ♂ 14 (acute rh. fever with carditis—after cortisone Rx)	"	−37, −29, 8	−19	8, 12, 74, 11, 28, 68, 77, 22	37
	B.P. ♂ 14 (Sydenham's chorea)	"	−3, −4, −23, 12, −21	−8	50, 7, 33, 36, 41	33
	C.E. ♀ 8 (Sydenham's chorea)	"	−1, 8, 64	24	50, 7, 33, 36, 41	33

* Difference in ascorbic acid concentration (mg/100 g fresh adrenal) between left adrenal, removed prior to injection of test material and the right adrenal, removed one hr after injection of test material.
† Days on cortisone. ‡ Days after cortisone withdrawal.

definitive statement regarding the exact concentration of ACTH in the blood of these patients, they do indicate that a greater than normal concentration of hormone is present. Samples of blood obtained from children with adrenogenital syndrome during cortisone

therapy (12.5 to 50 mg/day, intramuscularly or orally) did not contain detectable quantities of ACTH when assayed at doses equivalent to 20 and 40 ml. ACTH was not detectable in the blood of patients following cortisone withdrawal except in the case of E. Wi. In the miscellaneous group, 2 of 3 children with acute rheumatic fever and a child with spontaneous hypoglycemosis had unusually high concentrations of blood ACTH. In the case of J. B., cortisone therapy was followed by a return of the blood ACTH titer to an undetectable level. These findings in patients with rheumatic fever are interesting and justify further investigation.

Discussion. It is to be emphasized that the data presented are insufficient to permit definitive conclusions. In particular, the study lacks individual cases in which blood samples were analyzed in all three categories, *i.e.*, prior to, during, and after cortisone therapy. This type of control study is important since the degree of involvement of the adrenal cortex undoubtedly varies among patients in regard to cortical hormone output.

Blood from children in the control group contained no detectable ACTH. It may be estimated that the concentration of ACTH is less than 0.5 mu per 100 ml blood. These findings are in agreement with observations in normal adults(3) and in non-stressed intact rats(3).

In the untreated children with adrenogenital syndrome ACTH was present in the blood in detectable quantities. The limited results are compatible with the hypothesis of Bartter *et al.*(4) and of Wilkins *et al.*(5) that in the adrenogenital syndrome there is an increased rate of discharge of ACTH from the pituitary [See schema presented in Fig. 13 of the paper of Bartter *et al.*(4)]. The increased activity of the adenohypophysis is presumed to be secondary to a primary defect in the adrenal cortex. The hyperplastic adrenal gland produces an excess quantity of androgen but a relatively deficient quantity of cortical hormone. That the adrenal cortex is incapable of producing an optimal quantity of cortical hormone is suggested by the fact that the administration of ACTH fails to induce a significant increase in the titer of blood 17-hydroxycorticosteriods(6). Furthermore, the reduction in blood ACTH titer by cortisone is compatible with the concept that the primary defect is in the target gland and not in the adenohypophysis.

Blood ACTH was detectable in only one patient with adrenogenital syndrome in the post-cortisone group. This is difficult to explain in terms of the above concept since one would expect the titer of ACTH to rise following cortisone withdrawal. However, the dose of cortisone, the duration of therapy, and the time elapsed after withdrawal of therapy are variables which must be investigated in greater detail before definitive conclusions are made.

Summary. The oxycellulose technic has been applied to the analysis of blood ACTH in children. ACTH was not present in detectable quantities in the blood of afebrile children without endocrine disease. ACTH was detected in the blood of untreated, but not of cortisone-treated, children with adrenogenital syndrome.

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Effect of Cortisone Acetate on Experimental Rocky Mountain Spotted Fever in the Guinea Pig. (20223)

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(With the technical assistance of William Brooks.)

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In certain diseases, the administration of cortisone may relieve many of the symptoms and suppress the signs of illness. In spite of this apparent beneficial effect, cortisone therapy may be harmful in diseases such as pneumonia or tuberculosis, since it often accelerates the spread of the infection(1,2). It has been suggested, however, that in the therapy of Rocky Mountain spotted fever and typhoid fever, this hormone may be a valuable adjunct to chloramphenicol(3,4). Because of the intracellular position of rickettsiae as opposed to the extracellular location of most bacteria, one might expect a different response to cortisone in animals or subjects infected with the former microorganisms. The hormone might alter the rate of spread from the site of inoculation, the rate of growth within cells, or the response of the host cells.

The present study was undertaken in an effort to determine the effects of two different dosage schedules of cortisone acetate administered to guinea pigs with experimental spotted fever.

Material and methods. A total of 91 guinea pigs were used. The Bitter Root strain of *R. rickettsii* was passed serially by intraperitoneal injections. Whole bacteriologically sterile, citrated blood obtained by cardiac puncture, or a splenic emulsion in sterile physiologic saline solution was used as passage material. The animals from which passage material was obtained were sacrificed on either the fourth or fifth day of fever. Daily determinations of the body weight and the rectal temperature were made on all animals. Complement fixation tests were performed by a standard method(5). The significance of the difference in the mortality rate between the test and control groups was tested by the chi-square method(6).

Plan of the Experiment. Exp. 1 was designed to determine whether the daily administration of a dose of cortisone acetate comparable to a clinically therapeutic dose in human beings would influence the course of the infection. Five sets of observations were made upon 51 male animals. Each set of animals was given a different preparation of rickettsiae. In each set, the animals were divided into test and control groups of comparable body weight. All animals in the 2 groups were inoculated with the same volume of material containing the rickettsiae. Starting on the day of infection, each animal in the *test group* was given daily subcutaneous injections of 1 mg of cortisone acetate (a dose which is comparable to 150 mg for a 70 Kg man) for 17 days. The *control group* was not given cortisone. All observations were continued for 17 days after infection. *Exp.* 2, performed several months after completion of Exp. 1, was designed to determine whether the administration of a large dose of cortisone acetate for 5 consecutive days at the peak of the clinical illness would alter the mortality rate or the degree of fever. Forty guinea pigs were divided equally into 2 sets, which were studied one month apart. The 20 guinea pigs in each set were observed simultaneously and were given aliquots of a single inoculum of rickettsiae. Ten animals in each set were not given cortisone; the remaining 10 received 5 daily intramuscular injections of

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TABLE I. Effect of Cortisone Acetate, 1 mg Daily, on Mortality Rate in Guinea Pigs with Spotted Fever (Exp. 1).

Severity of infection	Total No. animals	Survivals	Deaths
Control group (*R. rickettsii* only)			
Mild, no fatality	3	3	0
Moderate	15	4	11
Severe, fatality rate over 90%	8	1	7
Total	26	8	18
Test group (*R. rickettsii* and cortisone)			
Mild, no fatality	3	3	0
Moderate	14	8	6
Severe, fatality rate over 90%	8	0	8
Total	25	11	14

cortisone acetate, 25 mg, starting on the morning of the fourth day of infection.

Results. Exp. 1. Sixty-nine per cent of the control animals infected with spotted fever and 56% of the test animals also given cortisone acetate died; the difference in the overall fatality rate was not significant. However, when the 5 sets of animals were arbitrarily divided into 3 groups, depending upon the severity of the infection, there was a suggestion that the administration of cortisone acetate did have a beneficial effect (Table I). All of the six animals with "mild" infections survived. None developed fever (rectal temperature of 104°F or greater). Complement-fixing antibody to spotted fever antigen was found in all animals on the twenty-second day of infection. This set of animals was given the original lyophilized material, which subsequently increased in virulence upon serial passage in guinea pigs. All but one of the 16 animals with infections classified as "severe" succumbed. There was no significant difference between the test and control groups as to the duration or height of fever or the day of death. In the 29 animals classified as having "moderately severe" infections, 43% of those given cortisone acetate and 73% of the control guinea pigs succumbed. The difference in the mortality rates in the two groups was statistically significant (P = 0.01 to 0.02). The mean duration of fever in the test group was 5.5 days, as compared with a mean of 4.1 days in the control group. In the test group, the average day of death was 11.5 days after inoculation; in the control group, 10.4 days. In general, the changes in body weight in the two groups paralleled each other.

Exp. 2. Eighty per cent of the animals in each group died during the period of observation. Only 6 of the 16 deaths in the test group occurred while the animals were receiving cortisone acetate, whereas 14 deaths occurred during the same period in the control group (Table II). Thus, most of the fatalities in the test group occurred after cortisone was discontinued. The mortality rate in the test group during the first 8 days of infection was significantly lower than that in the control (P = less than 0.01). The mean day of death in the infected animals not given cortisone acetate was 8.1 days; that in the test group which received the hormone was 9.9 days. A striking difference was noted clinically in the physical appearance of the test and control animals within 48 hours after the institution of cortisone therapy in the test group. Those given the drug were much livelier and showed less ruffling of the coat than those not receiving cortisone.

The mean daily decrease in body weight in the 2 groups was practically the same during the first 8 days. An abrupt fall in the mean daily rectal temperature occurred in the control group between the sixth and seventh days of infection, and all except 2 of the animals were dead by the ninth day. In the group given cortisone acetate, the highest mean temperature of 104.7°F was found on the fourth day of infection; it subsequently decreased gradually until the thirteenth day of infection.

Comment. Although the daily administration of 1 mg of cortisone acetate did not significantly alter the overall mortality rate in guinea pigs infected with *R. rickettsiae*, analysis of the results according to the severity of the disease suggested that the hormone may be of value if the infection is of moderate severity. The justification for such an arbitrary classification lies in the fact that the efficacy of a therapeutic agent which does not directly inhibit the growth of an infecting organism may not be apparent, either in the presence of a minimal infection without systemic reactions or in the presence of an over-

TABLE II. Effect of Cortisone Acetate, 25 mg Daily, on Period of Survival in Guinea Pigs Infected with *R. rickettsii* (Experiment 2). 20 animals in each group.

	Total deaths	Period of survival (days after infection) 6	7	8	9	10	11	12	13	14	15	16	17
Test (Given *R. rickettsii* and cortisone)	16	1	2	3	2	3	1	2		1			1
Control (Given *R. rickettsii*)	16		7	7	1						1		

whelming infection. In the presence of a moderately severe infection, when there is sufficient time for the body's defense mechanisms to be mobilized, any beneficial effect which the drug possesses may become evident. It has been observed that in guinea pigs with fatal infections of *R. rickettsii,* the temperature drops rather precipitously to subnormal values about 12 hours prior to death. The longer mean duration of fever in the group given cortisone acetate and classified as having moderately severe infections has therefore been attributed to a reduction in toxemia resulting from the administration of cortisone. The longer period of survival in the cortisone-treated animals is also compatible with this interpretation.

The observations in Exp. 2 appear to confirm the impression that cortisone acetate has a beneficial effect on the course of moderately severe cases of Rocky Mountain spotted fever. No deaths occurred in either group prior to the fourth day of infection, at which time the administration of large doses of cortisone acetate was begun in the test group. During the period of drug therapy, the number of deaths in the treated group was significantly smaller than that in the control group. In addition, the clinical appearance and behavior of the treated animals was less in keeping with a severe illness. Following discontinuation of cortisone, the number of deaths in the test group was greater than that in the control group, so that the overall mortality rate was identical. It appears, therefore, that the beneficial effects of cortisone acetate were transitory.

It is not possible from the present study to determine the exact mechanism of the protective effect apparently afforded by cortisone acetate in guinea pigs infected with *R. rickettsii.* There is no evidence to suggest that it may be due to a suppression of the growth of rickettsiae in the infected animals, and it has been previously observed that cortisone acetate does not affect the rate of growth of *R. rickettsii* in the yolk sac of embryonated chick eggs(7).

Summary. 1. The effect of 2 different dosage schedules of cortisone acetate on guinea pigs infected with Rocky Mountain spotted fever of varying degrees of severity has been investigated. The overall mortality rate in 25 animals given daily intramuscular injections of 1 mg of the drug, starting on the day each received, by intraperitoneal injection, an inoculum of rickettsiae, did not differ significantly from the mortality rate in infected guinea pigs which received no treatment. The severity of the infection induced by different preparations of rickettsiae varied considerably; when this factor was taken into consideration, it appeared that cortisone acetate in doses comparable to those employed in human beings reduced the mortality rate in those animals with moderately severe infections. 2. In another group of 20 animals which received daily intramuscular injections of 25 mg of cortisone acetate for 5 days at the peak of the illness (fourth through the eighth day of infection) the overall mortality rate was identical with that in 20 infected animals not given the hormone. During the period of administration of this relatively huge dose of cortisone acetate, however, the fatality rate was significantly lower than that in the untreated animals.

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Adipokinetic Activity of Oxycel-Purified Corticotropin.* (20224)

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The administration of crude extracts of anterior pituitary to mice, rats, and guinea pigs was found by Best and Campbell(1,2) to produce a rapid increase in the quantity of lipid in the liver and a decrease in carcass fat. It was subsequently shown that the source of the lipid which accumulated in the liver under these circumstances was the fat storage depots (3,4) and that the increment of hepatic lipid was composed largely of glycerides(5); it was concluded that pituitary extracts promoted the mobilization of depot fat to the liver. Since only a fraction (20-50 per cent) of the fat disappearing from the depots could be accounted for in the liver(2,5), the utilization as well as the migration of fat appeared to have been enhanced. The descriptive term adipokinin has been applied to the pituitary principle responsible for this metabolic effect(6). A number of studies have shown that pituitary extracts rich in one or another of the well-defined anterior pituitary principles (gonadotropins, corticotropin, thyrotropin, growth hormone) may produce various effects on fat metabolism of experimental animals, and it has sometimes been implied that one of these might be the pituitary factor causing these effects. Fatty liver and lipemia have been induced by thyrotropic preparations(7-9), and pronounced adipokinetic activity has been reported in preparations rich in growth hormone(10,11) and prolactin(12). Payne(13), studying a variety of crude and partially purified pituitary extracts, found no correlation between adipokinetic activity and that of any one of the well-characterized hormones, and concluded that the fat-metabolizing factor was a distinct principle.

In the present study estimates were made of the adipokinetic activity of some of the extracts obtained in the course of fractionation of hog anterior pituitary according to the procedures devised in this laboratory for the extraction and purification of corticotropin and growth hormone(14-18). The results indicate that a fraction potent in adipokinin is obtained by these methods and lend support to the idea that the fat-mobilizing principle is a factor distinct from other pituitary hormones.

Experimental. Assays were performed by a modification of the method of Campbell(19). Female mice of the Swiss Webster strain (obtained from Taconic Farms and local dealers) varying in weight from 18 to 24 g were used; in most experiments, mice of nearly uniform weight were selected. The animals were deprived of access to food one hour before pituitary extracts were to be administered, and after injection they were kept in cages containing water but no food. In the earlier experiments the test materials were injected subcutaneously in 0.5 cc volume at pH 4 and the mice were killed seven hours later; in later experiments, particularly when potent extracts

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were tested, the materials, dissolved in 0.9% saline solution adjusted to pH 9, were injected intraperitoneally in 0.2 cc volume and the animals were sacrificed 3 to 3½ hours later. Control mice, injected with saline solution alone, were used in each experiment. An impression was gained that the test was more sensitive and the results more reproducible when the injected material was given in alkaline rather than in acid solution. The mice were killed by skull fracture and exsanguinated by a cervical incision; in such bled animals one may roughly estimate the quantity of liver fat from the degree of yellowness of the liver. A portion of liver (470-520 mg) was removed, blotted, weighed and transferred to a Potter-Elvehjem homogenizing tube. The lipids were extracted by a modification of the method of Folch and his associates(20) by homogenizing the tissue in 15 cc of a mixture of chloroform-methanol (2:1 by volume). The homogenate was then filtered through Munktell's No. 00 filter paper and a 10-cc aliquot of the filtrate was transferred to a tared beaker and evaporated to dryness at 100-110°, the weight of the dried lipid then being obtained by difference. Results were expressed as percentage of lipid in the liver. In several preliminary experiments it was found that approximately 1.5 mg of the crude lipid extracted from 500 mg of liver was insoluble in petroleum ether, and therefore, as a correction for non-lipid materials in the lipid extract, 0.3 was arbitrarily subtracted in all experiments from the value obtained for the percentage of lipid, and the values for percentage lipid indicated in the tables and figures have been corrected in this way. The presence of non-lipid materials in lipid extracts of brain was noted by Folch *et al.*(20). The completeness of lipid extraction from the liver by the method used was indicated by the failure to extract by prolonged Soxhlet extraction (chloroform) significant quantities of lipid from previously ground, extracted liver residues.

Results. The following preparations were studied: Hog anterior pituitary powder (acetone dried). Preparation A, or crude corticotropin, was the material prepared by glacial acetic acid extraction of hog anterior pituitary as described by Payne, Raben, and Astwood (14) and designated by them "crude corticotropin." Preparation D, or oxycel-purified corticotropin, was the extract obtained by acid elution from oxycellulose which had been stirred with preparation A under the conditions devised by Astwood, Raben, Payne, and Grady (15). "Oxycel-unadsorbed material" refers to that part of preparation A which remained after the latter had been twice-treated with oxycellulose, and was the "acetic acid fraction" of Raben and Westermeyer(17). "Glacial acetic acid extracted growth hormone" was the preparation of growth hormone described by Raben and Westermeyer(17,18). Some experiments were also made with Armour growth hormone preparations, mainly lot No. R285128.

The data (derived from many experiments performed over a period of months) relating the concentration of hepatic lipid to the dose of extract administered seven hours previously is summarized in Fig. 1 for four preparations, which, in order of decreasing potency, were preparation D, preparation A, anterior pituitary powder, and the oxycel-unadsorbed material. Since the slopes of the curves were not uniform and the response was not linear over the entire dosage range, precise quantitative estimates of relative potency was difficult. However, if a comparison was made of the smallest dose which produced an average value of 10% for the hepatic lipid concentration (the control values for this series being 6.9%), the approximate effective dose for each preparation was: preparation D, 15 μg, preparation A, 500 μg, anterior pituitary powder, 2 mg, oxycel-unadsorbed material, 15 mg, indicating that preparation D was about 30 times as potent in adipokinin as preparation A, 150 times as active as the starting anterior pituitary powder, and 1000 times the oxycel-unadsorbed fraction. Since the corticotropic activity of preparation D has been found to be 40 times that of preparation A and 200 times the anterior pituitary powder while the oxycel-unadsorbed material is greatly depleted(16), the parallelism between adipokinetic and corticotropic activity in the fractionation procedure is apparent. Preparation D constitutes 0.25% by weight of the anterior pituitary powder serving as starting material, and approximate-

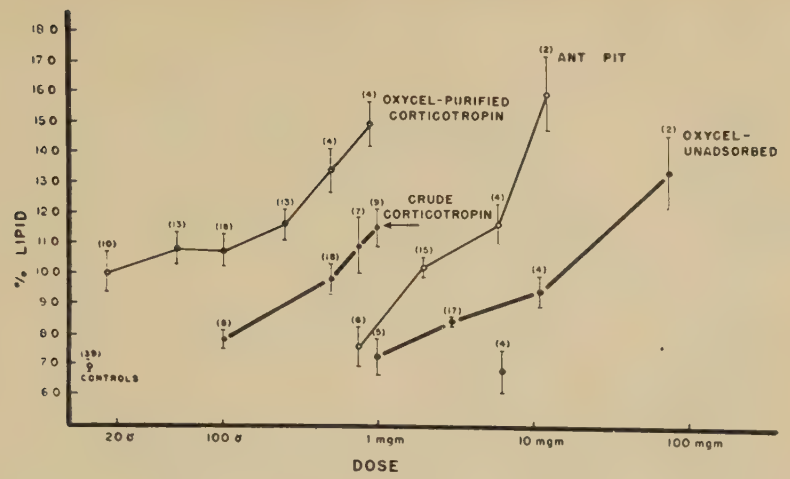

FIG. 1. Relationship between dose and response (% hepatic lipid 7 hr after injection) for 4 pituitary preparations. In this and the other figures the numbers in parentheses indicate the number of mice used to determine the mean represented by each point, and the limits of the vertical line through each point indicate the stand. error of the mean. The value for the control mice used in these experiments is shown in the lower left corner of the chart.

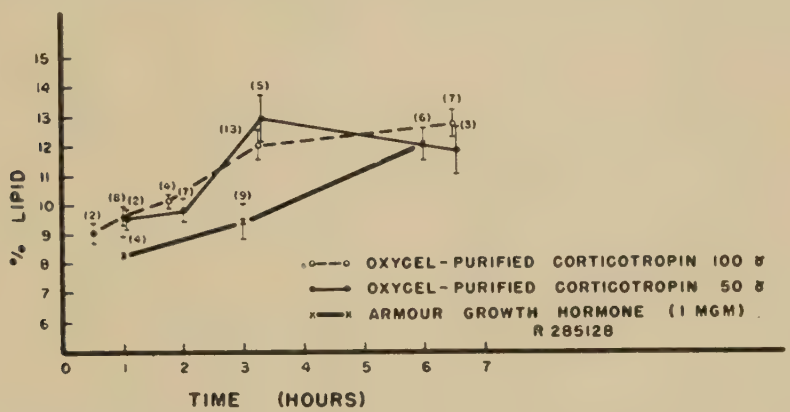

FIG. 2. The percentage of lipid in the liver at different times after injection of preparation D and Armour growth hormone. The extracts were administered intraper. in alkaline solution.

ly one-third of the adipokinin in the powder is recovered in Preparation D. The oxycel-unadsorbed fraction, greatly depleted of adipokinin, is used as starting material for the preparation of growth hormone by the procedure of Raben and Westermeyer; the growth preparation constitutes 12-15% of the total solids of the unadsorbed fraction and is equal in growth activity to the purified preparations currently in use(17,18). Even if all the adipokinin in the oxycel-unadsorbed fraction were concentrated in the growth hormone preparation the product would still be expected to be perhaps only 1% as active in adipokinin as preparation D, and adipokinetic activity of this order has been noted (Fig. 4).

That part of preparation A which was not adsorbed by oxycellulose in the quantity (8% by weight) used to obtain preparation D was found to be considerably more potent in adipokinin before than after it had been treated with a second, larger quantity of oxycellulose (20% by weight). The fraction eluted by hydrochloric acid from the second batch of oxycellulose was potent in adipokinin, perhaps half as active as preparation D. Corticotropin and intermedin remaining unadsorbed after the first oxycel treatment have similarly

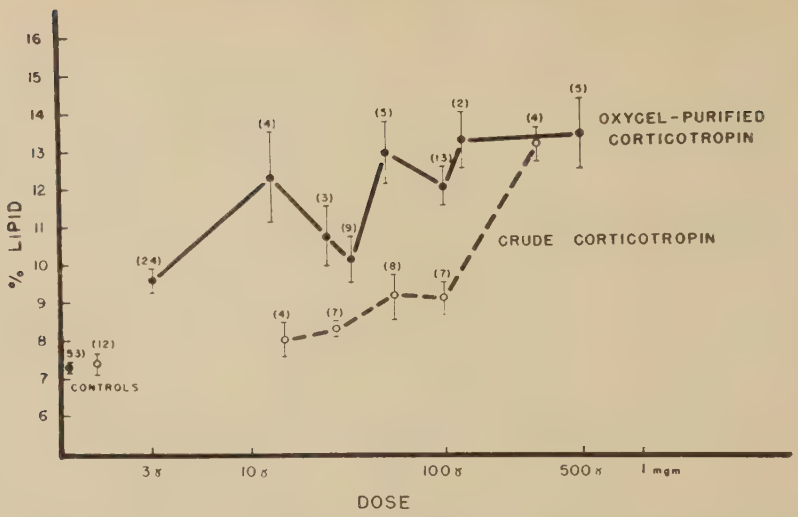

FIG. 3. Percent hepatic lipid 3 hr after administration of various doses of preparations A and D. The points are the average of separate experiments performed over a period of months and different lots of the preparations were used. Variation in the sensitivity of the animals and in the potency of the extracts may be factors in the variability of the results. The 4 values averaged to yield the point at 300 μg crude corticotropin were obtained in one experiment with the same batch of preparation A. Control values for each series are indicated on the left.

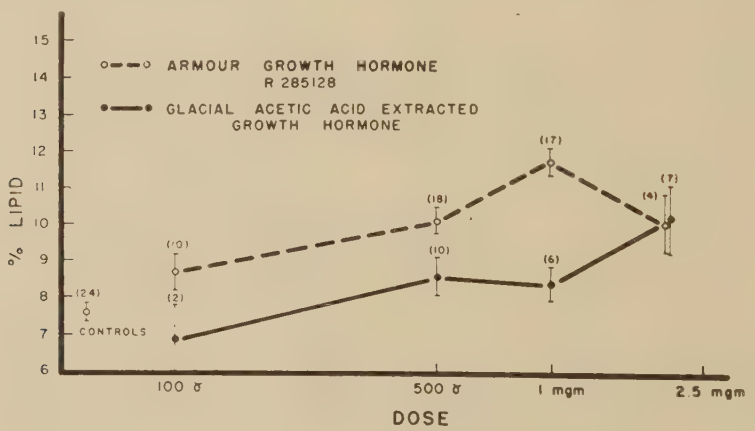

FIG. 4. Comparison of adipokinetic potency of Armour growth hormone and of Raben-Westermeyer growth hormone. Seven-hour test period.

been found to be extracted by retreatment with oxycel(21). The results of injecting mice with alkaline solutions of oxycellulose which had been stirred with preparation A and from which preparation D had been eluted, indicated that insignificant adipokinetic activity was retained by the adsorbent after acid elution.

The accumulation of lipid in the liver consequent to the intraperitoneal administration of preparation D was found to occur at a surprisingly rapid rate. Doses of 10-100 μg produced some increase over control values in hepatic lipid concentration within 30 to 60 minutes; the peak value was reached at about 3 hours and changed little between then and 7 hours (Fig. 2). With less potent adipokinetic extracts (Armour's growth hormone and oxycel-unadsorbed material) the accumulation of lipid was more gradual, the values 6 hours after injection being considerably higher than at 3 hours. It is not clear whether this difference between the stronger and weaker preparations is attributable to factors such as different rates of absorption and duration of action or

whether there is a qualitatively different mechanism of fat accumulation. The 3-hour period is convenient for the comparative assay of potent preparations. The 3-hour values for liver lipid concentration obtained with various doses of preparation A and D are shown in Fig. 3, and the results may be interpreted as showing approximately the same order of comparative potency as the 7-hour tests.

Direct comparisons were made of the adipokinetic activity of 2 growth hormone preparations and of preparation D with the results shown in Fig. 2 and 4, which indicate that adipokinin is not growth hormone. The data of Fig. 2 show that at 6 hours 50 μg preparation D and 1 mg Armour growth hormone (R285128) caused approximately the same increase in liver fat, while at 3 hours, in the same doses, preparation D was more potent than the growth hormone preparation. Fig. 4 illustrates the adipokinetic activity at several doses of 2 pituitary extracts of equal growth activity, the Armour preparation and glacial-acetic acid extracted growth hormone(18); the former preparation would appear to be about 5 times the latter in fat mobilizing activity.

Since the most active adipokinin preparation studied, preparation D, is rich in corticotropin and intermedin(21), the possibility that the fat-mobilizing factor is identical with one of these must be considered, as well as the possible modification of the adipokinetic by the corticotropic effect. Preparation D did not produce an increase in the liver lipid of the adrenalectomized mouse, but if such animals were maintained postoperatively with cortisone, (0.5 mg cortisone acetate subcutaneously daily), the adipokinetic response to preparation D was observed, confirming the observations made with other pituitary extracts that the fat-mobilizing effect is not mediated by the adrenal but requires the presence of cortical hormones(13,22). The comparative adipokinetic potencies of preparation D, Armour growth hormone, and the oxycel-unadsorbed material, as judged by assays on adrenalectomized mice maintained with cortisone, were of the same order as in the intact animal (Table I), suggesting that adipokinetic assays in intact animals were not seriously

TABLE I. Adipokinin Effects in Adrenalectomized Mice.

	Preparation and dose		No. animals	% lipid in liver ± S.E.
A	Preparation D	100 μg	(11)	9.9 ± .35
	Controls	0	(11)	6.4 ± .32
B	Preparation D	50 "	(3)	11.4 ± .66
	Armour growth hormone	500 "	(3)	10.2 ± .36
C	Preparation D	25 "	(2)	9.1 ± .24
	Oxycel–unadsorbed material	3 mg	(3)	7.5 ± .05

Mice adrenalectomized 2-3 days before injection with pituitary extracts and post-operatively received 0.5 mg cortisone acetate daily subcut. up to and including the day the tests were made. The data of B and C were derived from single experiments; that of A from a number of separate experiments. Seven-hr test period.

modified by the corticotropin of preparation D. It has been reported that pre-treatment of mice with cortisone enhances the fat mobilization induced by some growth hormone preparations(22). In the present study the subcutaneous injection of 0.5 mg cortisone acetate once daily for 3 days did not appreciably change the percentage of fat (7-hour assay) in the livers of intact mice injected with oxycel-unadsorbed material, an extract containing growth hormone, as compared with control animals not receiving cortisone.

Intermedin and adipokinin do not appear to be the same principle. A partial separation of the intermedin and adipokinetic activities of preparation D may be made by the use of chromatographic columns of charcoal and of cation-exchange resin(23,24). The non-identity of the two principles has been further suggested by the results obtained on assay of the intermedin and fat-mobilizing activity of preparation D which had been heated in a boiling water bath for 2 to 3 minutes at pH 13, and then cooled, neutralized, diluted appropriately and injected into frogs and mice. The intermedin potency of preparation D treated in this way was found to have been greatly enhanced, while adipokinetic potency had been uniformly decreased (Table II). In its instability under these conditions adipokinin resembles corticotropin(25).

Solutions of preparation D in 0.1 N HCl

TABLE II.
Effect of Heat and Alkali on Adipokinin. 3 exp.

Preparation D	Dose (μg)	No. of mice	Hepatic lipid (%) ± S.E.
Heated	70	2	8.3 ± .65
Unheated	70	2	11.1 ±1.9
Heated	250	2	9.7 ± .60
Unheated	250	2	14.5 ± .65
Heated	3	4	**9.1 ± .47**
Unheated	3	3	11.9 ± .60

Preparation D in 0.1 N NaOH heated in boiling water bath 2-3 min., cooled, neutralized, diluted, and injected in mice. Unheated material handled in same manner except not heated. In exp. 3, 0.15 μg doses of heated and unheated materials were injected in intact frogs; heated material caused maximal darkening, while unheated preparation at this dose caused minimal darkening.

kept at 5°C for several months have shown no change in adipokinetic potency during this period, and no loss of potency was detected in one week at room temperature, pH 8. Incubation of preparations A and D in 0.1 N HCl with pepsin at room temperature for 18 to 24 hours, the ratio of enzyme to extract being 1/30 to 1/80, led to marked loss of activity, as did incubation of preparation D with trypsin at pH 8 for 18 hours at room temperature. Large doses of preparation D (2-8 mg) administered to mice by stomach tube resulted in a marked increase in the hepatic lipid concentration at seven hours; smaller doses were only irregularly effective by the oral route. The oral administration of glucose (0.5 cc, 50% solution) immediately before injecting mice with preparation D usually lessened the quantity of lipid accumulating in the liver at 3 or 7 hours, but often the difference was small, compared with controls not receiving glucose, and in no instance was the accumulation of lipid in the liver entirely prevented.

Discussion. The results support the view that the fat-mobilizing agent of the pituitary is a distinct principle. The marked adipokinetic activity of preparation D, together with the fact that this preparation contains no detectable thyrotropin, luteotropin, or gonadotropin(21), would tend to exclude these principles as the fat-mobilizing factor and evidence suggesting that neither growth hormone nor intermedin is responsible for the adipokinetic effect has been indicated above. It is more difficult to determine whether adipokinin and corticotropin are not identical, particularly since these activities tend to accompany each other in the glacial acetic acid-oxycellulose fractionating procedure. Some pituitary preparations which have been reported to cause fat mobilization have had little corticotropic activity(10,13). Since the administration of cortisone does not reproduce the fat mobilization observed to result from preparation D injections, and since the adipokinetic activity of preparation D is independent of the presence of the adrenal glands, if the adipokinetic activity were inherent in corticotropin, it presumably would have to be by virtue of an extra-adrenal action of the latter. It would appear unlikely that 2 such diverse phenomena as mobilization of depot fat and stimulation of the adrenal cortex should be attributable to the same substance, but decision as to the nonidentity of adipokinin and corticotropin must await the complete separation of these activities from one another.

Summary. Purified corticotropin prepared from hog anterior pituitary by the glacial acetic acid-oxycellulose method was found to be potent in adipokinin. Doses of 3 to 15 μg produced an increase of 40% in the hepatic lipid concentration of mice within three hours after injection. Pituitary adipokinin appears to be a principle distinct from the gonadotropins, thyrotropin, corticotropin, growth hormone, and intermedin.

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Effects of Total Body X-Irradiation on Some Constituents of Liver and Kidney of the Rat. (20225)

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To characterize certain metabolic alterations in the liver and kidney due to x-irradiation, phosphate distribution, glycogen, sulfhydryl, sodium, potassium, and water content as well as cytochrome oxidase activity of these organs were studied in rats subjected to lethal total body x-irradiation.

Inasmuch as the content of some of these constituents is known to be influenced by the secretion of the adrenals, some of these studies were done on adrenalectomized animals. Since decreased food intake is part of the post-irradiation syndrome, the effect of fasting alone on the above tissue constituents was also studied.

Materials and methods. Male Sprague-Dawley rats, weighing 235-280 g and fed Purina chow diet, were used throughout. The adrenalectomized rats were maintained on 0.95% NaCl drinking solution and used about 10 days after operation. Radiation dosage was 1000 r in the experiments on phosphate distribution in the liver and kidney, and on cytochrome oxidase activity in the liver; 880 r in the other experiments. Radiation factors were: 200 kv, 6 ma, ½ mm Cu 1 mm Al filter, target distance approximately 29 cm and 40 r/min dosage rate measured in air.* The majority of deaths occurred within 6 to 14 days after irradiation with 880 r and 4 to 6 days after irradiation with 1000r. A. *Phosphate Distribution.* Phosphate determinations on the tissue were made according to the method of Umbreit(1) except that the animals were killed by decapitation and the tissues were quickly removed, frozen in a dry ice-acetone bath, and lyophilized. Trichloroacetic acid extracts of the tissues were prepared and the barium-insoluble fraction was analyzed for inorganic phosphorus and easily hydrolyzable phosphorus(15 min., 100°C, 0.2 N HCl). All results are reported as mg P/100 g dry tissue. The irradiated animals, which had access to food and water, were sacrificed 1 to 6 days after irradiation. Food, but not water, was withheld overnight prior to sacrifice. B. *Liver Glycogen and Blood Glucose.* Food was withheld for periods indicated in Table II, while water or 0.95% NaCl solution (adrenalectomized animals) was allowed *ad libitum.* The animals were sacrificed by decapitation, and samples weighing approximately 1 gram were removed from the median lobe of the liver and immediately immersed in cold (3° to 5°C) saline. The samples were then cut into 6 nearly equal parts under cold saline, blotted on filter

* The authors wish to express their appreciation to the Radiobiology Department of this Laboratory for advice and assistance in the irradiation procedure.

TABLE I. Phosphorus Distribution of Liver and Kidney of X-Irradiated Rats. Dosage 1000 r. Average values of dry tissue ± S.D.

	Liver			Kidney		
	No. of rats	Inorganic P, mg/100 g	Labile P, mg/100 g	No. of rats	Inorganic P, mg/100 g	Labile P, mg/100 g
A. Control	21	23.7 ± 4.3	79.8 ±10.3	25	32.5 ± 6.7	95.3 ± 9.6
B. Days after irradiation						
1	6	16.0 ± 3.8	60.0 ± 4.7	6	34.7 ± 7.3	106.6 ± 6.4
2	7	14.5 ± 1.8	55.2 ± 9.0	8	32.4 ± 8.4	99.8 ± 8.6
3	5	19.6 ± 4.4	54.2 ± 9.8	6	35.4 ± 5.9	97.2 ± 8.5
4	10	20.7 ± 6.3	64.2 ±17.2	10	36.7 ± 7.5	95.2 ± 6.8
5	8	24.0 ±10.5	80.2 ±12.1	8	29.3 ± 9.0	101.4 ± 8.4
6	5	19.6 ±11.2	84.4 ±17.3	6	24.3 ± 9.7	99.2 ± 5.6
C. No. of days without food						
3	3	21.5 ± 2.4	80.0 ± 7.2	3	28.7 ± 3.5	103.1 ± 4.1
4	3	16.9 ± 2.4	74.4 ± 4.6	3	28.7 ± 2.1	97.9 ± 4.5
5	3	19.7 ± 0.6	66.6 ± 5.8	3	34.2 ± 3.8	97.0 ± 3.7

TABLE II. Effect of Total Body X-Irradiation (880 r) on Liver Glycogen in Rats.

		No. of animals	Treatment	Liver glycogen, %
Normal				
Control	Food & water *ad lib.*	5	0	5.33 ± .15*
	Fasted 24 hr	23	0	.10 ± .05
Irradiated	" 24 " after	12	0	2.08 ± .74
	" 24 " before & after	7	0	2.12 ± .50
Adrenalectomized				
Control	Fasted 24 hr	2	0	.09 ± .01
Irradiated	" 24 " after	4	0	.39 ± .17
Control	" 48 "	2	0	.01 ± .00
Irradiated	" 48 " after	4	0	.39 ± .44
Control	" 24 "	4	ACE†	.51 ± .22
Irradiated	" 24 " after	3	ACE†	4.44 ±3.37

* Mean ± S.D.

† Upjohn Adrenal Cortex Extract inj. in 5 doses of 1 ml each for 24 hr, last injection 1 hr prior to irradiation or beginning of fast.

paper, and placed in 30% KOH. Glycogen was extracted according to the method of Good, Kramer, and Somogyi(2), as modified by Sjogren, *et al.*(3), and determined by the anthrone method of Morris(4). Blood samples were collected in oxalated beakers from the point of decapitation and analyzed for glucose according to the method of Somogyi(5). C. *Sulfhydryl Content.* The sulfhydryl content of the liver and kidney was determined by a modification of the amperometric method of Benesch and Benesch (6). A rotating platinum electrode was employed as described by Herbert and Denson (7). Since certain difficulties were encountered in the titration with the use of sulfosalicylic acid (5 ml of a 22% solution for 0.5 g tissue sample), the protein precipitant was changed to 0.1 ml of a 10% solution of phosphomolybdic acid. No difference was found in the results employing either protein precipitant. For 16 hours before and throughout the experiment, the animals were fasted but were allowed water or 0.95% NaCl (adrenalectomized animals); they were sacrificed by decapitation. D. *Na, K and H_2O Content.* Tissue water content was determined by drying the samples to constant weight at 100°C. The Na and K contents were determined according to the procedure of Robertson and Peyser(8) using a Beckman flame photometer. The data presented are not corrected for the blood content of the tissue. For 16 hours before and throughout the experi-

ment, the animals were fasted but were allowed water or 0.95% NaCl (adrenalectomized animals); they were sacrificed by the administration of ether. E. *Cytochrome Oxidase.* Cytochrome oxidase activity was determined according to the method of Schneider and Potter(9). The irradiated animals, which had access to food and water, were sacrificed by decapitation 1 to 6 days after irradiation. Food but not water was witheld overnight prior to sacrifice.

Results and discussion. A. *Phosphate distribution.* The procedure employed here for the determination of labile phosphate (ATP) seemed to give higher values for the labile organo-phosphates than those previously reported. As early as 1 day after irradiation both inorganic and labile phosphate (ATP) in the liver were subnormal (Table I). The liver inorganic phosphate remained at this low level through the third day after irradiation and the ATP-phosphorus continued to decline slowly. On the fourth day, both inorganic and ATP-phosphorus in the liver rose, the inorganic phosphorus approaching the control level where it remained until death. The ATP-phosphorus, however, reached the control range on about the fifth day after irradiation and apparently continued to rise slowly. Fasting of 3 days duration did not produce any pronounced changes in the inorganic and labile phosphate of the liver (Table I). However, 4 and 5 days fasting caused a decrease of inorganic and labile phosphorus. It seems, therefore, that the radiation was responsible for the decrease in inorganic and labile phosphorus observed in the liver during the first 3 days after irradiation and for the increase in labile phosphate during the terminal stage. The fall in the ATP content following irradiation may be linked with the presence of increased glycogen. The increase of ATP content of the liver during the terminal stage may indicate a reduced glycogenetic capacity of the liver of the irradiated animals. In the kidney, the changes which occurred in the inorganic and labile phosphate content were not significant (Table I). Fasting of 3 to 5 days duration also did not produce any significant changes in the inorganic and labile phosphorus content of the kidney (Table I).

B. *Liver Glycogen and Blood Glucose.* The blood sugar in the irradiated animals fasted 24 hours after irradiation was found to be definitely elevated, 91 mg %, compared with 70.5 mg % for the control fasted animals. A similar observation has been made by Kohn (10).

As can be seen from Table II, the amount of hepatic glycogen was higher in the irradiated than in the control non-irradiated animals. After 24 hours of fasting, liver glycogen values were 2% as compared with 0.1% in the non-irradiated control group. These observations are in agreement with similar findings of other investigators(11-13). In adrenalectomized animals qualitatively similar results were obtained (Table II). To test the ability of the x-irradiated rats to form liver glycogen, animals were fasted 24 hours prior to irradiation and the fasting continued for an additional 24 hours. In striking contrast to the non-irradiated control fasting animals, the x-irradiated animals had a liver glycogen content up to 2% (Table II). It is likely that this production of glycogen is the result of an increased gluconeogenesis stimulated by the adrenals. It has been found by Patt and his associates(14) that total body x-irradiation (900 r) of rats resulted in a considerably increased functional activity of the adrenal cortex. However, the increased cortical activity alone cannot account for the observed liver glycogen accumulation since adrenalectomized animals also show some liver glycogen accumulation under these conditions (Table II). In this connection reference may be made to the observation that normal fasting rats, subjected to low pressures, will show an increase in the glycogen content of the liver(15). Apparently, total body x-irradiation impairs the mechanism for the physiological mobilization of liver glycogen. Further studies will be necessary to elucidate the cause of the impairment of glycogenolysis in the irradiated animals.

C. *Sulfhydryl Content.* The normal control value for liver, average 175 mg GSH/100 g tissue (Table III), agrees well with that reported by Peterson, Beatty and West(16) and by Ingbar, Otto, and Kass(17). Apparently, reduced glutathione is the principal acid-

TABLE III. Effect of Total Body X-Irradiation (880 r) on Sulfhydryl Content of the Liver in the Rat.

Control animals			X-irradiated animals starved 16 hr		
Sacrificed after hr starvation	No. of animals	Non-protein SH, mg GSH/100 g	Sacrificed hr after irradiation	No. of animals	Non-protein SH, mg GSH/100 g
16—SSA*	8	177 ± 14†	2	6	185 ± 18
16—PMoA‡	4	171 ± 22	4	4	208 ± 9
40	7	177 ± 17	12	9	219 ± 21
88	7	215 ± 12	24	10	239 ± 33
			72	8	263 ± 23
40—A§	4	137 ± 9	24—A	5	87 ± 13

* SSA—Sulfosalicylic acid (used as protein precipitant). † Mean ± S.D. Test for significance: In normal rats 1 day post-radiation "p" is less than 0.1, in adrenalectomized rats 1 day post-radiation "p" is less than 0.2. ‡ PMoA—Phosphomolybdic acid (used as protein precipitant). § A—Adrenalectomy.

soluble non-protein SH compound in the liver and kidney extract, since the values obtained by the amperometric method, employed in these studies, agree well with those reported by Woodward who employed a manometric method specific for reduced glutathione(18). Table III shows an increase in the sulfhydryl content of the liver following x-irradiation. Peterson, Beatty and West(16) found no change in the glutathione level of the liver in rats before the sixth day following 500 r total body x-irradiation, and a lowering between 6 to 19 days post-irradiation. The discrepancy in the results is probably due to the different x-ray dosage employed. An explaantion for the rise in the hepatic non-protein SH, observed after irradiation, can only be speculative at this time. An increased breakdown of glutathione-containing proteins, due to increased functional activity of the adrenal cortex observed after total body x-irradiation (14), may occur under these conditions. In addition, denaturation of cell proteins caused by the radiation, may increase the rate of proteolysis. In this connection reference may be made to the findings of Barron and his associates(19) that sulfhydryl-containing enzymes are readily oxidized by radiation and that it is possible to reactivate such enzymes after moderate radiation doses (100-200 r) on addition of a reducing agent. However, larger doses of x-irradiation were found to produce irreversible inactivation, which was attributed to protein denaturation. In contrast to normal animals, a reduction in the sulfhydryl content of the liver was observed in adrenalectomized animals after total body x-irradiation (Table III). The reasons for this difference in response are, at present, obscure. The absence of the adrenals may inhibit the rate of protein breakdown sufficiently to account for the observed effects. In contrast to the changes in GSH values found in the liver, no significant change was found in the sulfhydryl content of kidney after radiation; the control value (7 animals fasted overnight) was 144 ± 26 mg GSH/100 g tissue as compared with 158 ± 23 mg GSH/100 g tissue found 24 hours after irradiation (8 animals).

D. *Na, K and H_2O Content.*† The finding that there was no significant effect of radiation upon the sodium, potassium or the water content of the liver and kidney up to 48 hours after irradiation with perhaps the exception of a slight over-all increase in sodium in the kidney, agrees with similar observations of Bowers and Scott(20,21).

E. *Cytochrome Oxidase.*† Only negligible changes were found in the activity of cytochrome oxidase in the liver after lethal total body x-irradiation. This finding agrees with similar observations of other investigators who have studied the effect of total body x-irradiation on various other enzymes(22-24).

It is probable that several mechanisms are responsible for the changes in certain constituents of the liver and kidney observed after lethal total body x-irradiation. The nature

† Presentation of the experimental data is omitted since they only confirm the findings of other investigators. For details, see: Army Medical Research Laboratory Report No. 104, 20 Nov. 1952.

of these mechanisms is, at present, still rather obscure, and further studies are necessary for its elucidation.

Summary. 1. Lethal total body x-irradiation (1000 r and 880 r) of rats produced the following changes in certain constituents of the liver or kidney. 2. As early as 1 day after irradiation, both inorganic and labile phosphate (ATP) in the liver were definitely subnormal. The liver inorganic phosphorus remained at this low level through the third day after irradiation while the labile phosphate continued to fall slowly. On the fourth day, both inorganic and labile phosphate in the liver rose; the inorganic phosphorus approached the control level where it remained until death. The labile phosphate, however, reached the control range on about the fifth day after irradiation and apparently continued to rise slowly until death. In the kidney, the changes which occurred in phosphate distribution were not pronounced. 3. The fasting of rats immediately following total body x-irradiation resulted in what appeared to be a retarded rate of glycogenolysis. After 24 hours fasting, following radiation, liver glycogen values were 2% as compared with 0.1% in the fasted non-irradiated group. In adrenalectomized animals the liver glycogen content was also found to be higher after irradiation and fasting than the liver glycogen content found after fasting only. 4. A definite increase in the sulfhydryl content was found in the liver of fasted x-irradiated animals as compared with non-irradiated fasted animals. Removal of the adrenals produced a decrease in the sulfhydryl content of the liver of fasted animals. It was further reduced by irradiation. No significant changes were observed in the sulfhydryl content of the kidney of normal rats after irradiation. 5. There was no significant effect of x-irradiation upon the sodium, potassium or the water content of the liver and kidney up to 48 hours after irradiation with perhaps the exception of a slight increase in the sodium in the kidney. 6. Liver cytochrome oxidase activity showed only slight variations from the time of radiation exposure to subsequent death. 7. The possible influence of fasting on these liver and kidney constituents has been considered.

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Generalized Shwartzman Reaction.* V. Intravenous Injection of Colloidal Iron or Carbon on Response of Rabbits to Meningococcal Toxin. (20226)

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Treatment with cortisone was shown(1) to cause a marked alteration in the response of rabbits to a single injection of gram-negative bacterial toxin. An intradermal injection of meningococcal or *S. marcescens* toxin resulted in areas of hemorrhage and necrosis at the injected skin site, with histological resemblances to the Shwartzman reaction, in place of the usual reaction of edema and erythema produced by intradermal toxin in normal animals. Similarly, a single intravenous injection of toxin in cortisone-treated rabbits was followed by the development of bilateral cortical necrosis of the kidneys. This nephropathy is the identifying lesion of the generalized Shwartzman reaction, and occurs in normal rabbits only when 2 successive injections of toxin are given intravenously(2). Because of the possibility that the action of cortisone might be a manifestation of interference with protective functions of the reticuloendothelial system, the effects of thorotrast (colloidal thorium dioxide) and trypan blue on the response of rabbits to bacterial toxin were investigated (3,4). It was found that when an intravenous injection of either of these materials was followed within several hours by an injection of meningococcal or *S. marcescens* toxin, extensive hemorrhagic necrosis indistinguishable from the typical dermal Shwartzman reaction occurred at the injected skin site. When toxin was injected by vein following thorotrast or trypan blue, bilateral cortical necrosis of the kidneys and death occurred in a high proportion of animals. The occurrence of dermal hemorrhage and increased mortality after the injection of *S. marcescens* toxin in rabbits treated with thorotrast has also been described by Bennett(5).

The present report deals with the capacity of two other colloidal materials, known to be taken up by cells of the reticuloendothelial system after intravenous injection, to cause a similar alteration in the response to meningococcal toxin. These materials are colloidal carbon in the form of India ink, and colloidal iron, in the form of saccharated iron oxide.§

Materials and methods. Hybrid albino rabbits of both sexes, weighing 1.0-1.5 kilos, were used in all experiments; the animals were maintained on a diet of Purina rabbit pellets and water. All intravenous injections were given in the marginal ear vein; the intradermal injections were made in the shaved skin of the lateral abdominal wall. The toxin used was derived from a strain of meningococcus (44-B), obtained from Dr. Gregory Shwartzman. The method for preparing toxin has been described in an earlier communication(2). Various dilutions of toxin were made in sterile physiological saline, and the doses of toxin are indicated in the text by the dilution employed. The volume of toxin for intravenous injection was 2 cc; for intradermal injection, 0.25 cc. *Colloidal saccharate of iron oxide,* designated by the trade name of Proferrin, was obtained from Sharp and Dohme, Inc. A single lot of this material (1611K), containing 20 mg of iron per cc, was used in all experiments. It was injected intravenously, in doses of 1 or 2 cc per kilo. The *colloidal carbon* preparation consisted of a 5 or 10% saline suspension of Higgins American India ink. It was injected intravenously, in an amount of 5 cc per kilo.

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§ The use of this material was suggested by Dr. Jules Freund.

TABLE I. Occurrence of Hemorrhagic Dermal Necrosis and Renal Cortical Necrosis in Rabbits Receiving Intravenous Colloidal Iron and Intradermal Meningococcal Toxin. 4 rabbits in each series.

Treatment	Dilution of toxin*	No. with skin hemorrhage	No. with renal cortical necrosis
Colloidal iron†	undiluted	3	1
	1-10	0	1
None	undiluted	0	0
	1-10	0	0

* 0.25 ml of dilution indicated was given intradermally.

† Saccharated iron oxide (Proferrin, Sharpe & Dohme) injected intravenously 1 cc/k.

Experimental. Effect of colloidal iron on reaction to intradermally injected toxin. Eight rabbits were given an intravenous injection of saccharate of iron oxide, in an amount of 1 cc/k. Immediately thereafter, an intradermal injection of 0.25 ml of undiluted meningococcal toxin was administered to 4, and a 1-10 dilution of toxin to the other 4. A similar group of untreated animals received the same amounts of toxin. Observations of the skin site were made at frequent intervals, and at the end of a 36-hour period the survivors were sacrificed. The results are shown in Table I.

In the 4 animals given colloidal iron and undiluted toxin, the site of injection showed no visible reaction, except for slight blanching, for approximately 18 hours. Petechial hemorrhages then appeared in the skin of 3 rabbits, and during the next few hours these spread and coalesced, forming a circular area of deep blue hemorrhagic necrosis 3 or 4 cm in diameter. The lesions were similar to those previously observed in rabbits given intradermal toxin after an injection of thorotrast or trypan blue(4). In the untreated control animals, the intradermal injection of toxin caused a vigorous local reaction of erythema and edema, but no hemorrhages occurred in the skin.

The animals receiving a 1-10 dilution of meningococcal toxin did not develop hemorrhagic reactions in the skin. However, one of the treated rabbits died on the day following injection, and was found to have bilateral cortical necrosis of the kidneys. A similar lesion was present in one of the rabbits receiving undiluted toxin. Similar observations, indicating absorption of toxin from the skin, were previously made in rabbits treated with cortisone(1) and thorotrast(4).

Effect of colloidal iron on reaction to meningococcal toxin injected intravenously. Several groups of rabbits were given an intravenous injection of colloidal iron saccharate, in a dose of 1 or 2 cc per kilo. Six hours later various dilutions of menincococcal toxin were injected intravenously. Control animals, receiving colloidal iron alone or toxin alone, were included in the experiment. The results are shown in Table II. a) In the animals receiving the 1.0 cc dose of colloidal iron, bilateral renal cortical necrosis was produced by toxin in dilutions as high as 1-640. Most of these animals died within 12 to 24 hours after the injection of toxin. b) In the group of rabbits given iron saccharate in a dose of 2.0 cc per kilo, the reaction to intravenous meningococcal toxin was more rapid and severe. Most of the animals became prostrated within an hour after toxin, and died during the next 4-6 hours. The development of renal cortical necrosis was observed in only one rabbit, which received the 1-1280 dilution of toxin. The potentiation of the lethal effect of toxin was comparable to that previously encountered in rabbits treated with thorotrast before the injection of meningococcal toxin(4). c) In a group of control

TABLE II. Bilateral Cortical Necrosis of Kidneys and Death in Rabbits Injected Intravenously with Meningococcal Toxin Following Colloidal Iron.

Iron* oxide	Dilution of toxin†	No. of rabbits	No. dead	No. with renal cortical necrosis
1 ml/k	1:80	4	2	4
	1:160	4	3	1
	1:320	4	3	3
	1:640	4	1	1
	1:1280	4	0	0
	—	4	0	0
2 ml/k	1:160	3	3	0
	1:320	12	11	0
	1:640	3	3	0
	1:1280	4	2	1
	—	4	0	0

* Saccharated iron oxide (Proferrin) given intravenously 6 hr prior to injection of meningococcal toxin.

† 2.0 ml of saline dilution indicated given intravenously in each case.

TABLE III. Production of Hemorrhagic Necrosis in Skin of Rabbits by Meningococcal Toxin in Rabbits Injected with Colloidal Carbon.* 4 rabbits in each series.

Dilution of toxin*	No. with skin hemorrhage	No. with renal cortical necrosis
1:2	3	0
1:4	4	0
1:8	0	0

* 5 cc of a 5% suspension of Higgins American India Ink in physiological saline, inj. intrav. at time of intradermal inj. of toxin.

† 0.25 cc of meningococcal toxin, in dilution indicated, given intradermally in shaved skin.

Table IV. Production of Renal Cortical Necrosis in Rabbits by Intravenous Injection of Meningococcal Toxin Following Intravenous Administration of Colloidal Carbon.*

No. rabbits	Dilution of toxin†	No. dead	No. with renal cortical necrosis
6	1:80	0	4
6	1:160	1	4
5	1:320	1	1
6	1:640	2	1

* Colloidal carbon (Higgins American India Ink_R), 5.0 ml of 10% suspension in saline inj. intrav. 4 hr prior to injection of toxin.

† 2.0 ml of saline dilution of toxin indicated intrav.

animals given saccharate of iron oxide alone, in doses of 1 or 2 cc per kilo, no deaths or renal lesions occurred. Other control rabbits, given similar injections of toxin without colloidal iron, showed no reactions. When colloidal iron was injected 4 or 6 hours *after* the toxin, instead of before, the rabbits remained well.

Although the preparation of iron saccharate used in these experiments was apparently nontoxic in the doses employed, larger amounts of this material were found to cause death. For example, 3 of 6 rabbits given a dose of 4 cc per kilo by vein died within 24 hours. No kidney lesions were present in these animals by gross or microscopic examination, and no explanation is available for the lethal effect.

Effect of colloidal carbon on reaction of intradermally injected meningococcal toxin. Twelve rabbits were given 5 cc of a 5% suspension of India ink intravenously and, immediately thereafter, an intradermal injection of 0.25 cc of meningococcal toxin. Observations of the skin site were made, and 24 hours later all the animals were sacrificed. The results are shown in Table III. Three of four rabbits in the group receiving 1-2 toxin, and all of those given 1-4 toxin, developed hemorrhagic skin lesions which were similar to those encountered in the animals receiving colloidal iron. No deaths or renal lesions occurred in these animals.

Effect of colloidal carbon on reaction to meningococcal toxin injected intravenously. Groups of rabbits were given 5 cc of a 10% colloidal carbon suspension by vein, followed four hours later by various dilutions of meningococcal toxin. The survivors were sacrificed 24 hours after the injection of toxin. The results are indicated in Table IV. Renal necrosis occurred in all groups, most frequently in those receiving the 1-80 and 1-160 dilutions of toxin. Death occurred within less than 24 hours in some of the rabbits, but the lethal effect of toxin was less marked than in the animals which received colloidal iron followed by toxin (Table II). *Reversal* of the order of injection of the two substances, *i.e.* toxin followed by colloidal carbon, resulted in no deaths or instances of renal cortical necrosis. Other control animals given similar injections of toxin alone, or colloidal carbon alone, showed no ill effects.

Discussion. It has been shown that when rabbits are given intravenous injection of colloidal saccharate of iron oxide, or of colloidal carbon, an alteration of the reactivity to meningococcal toxin is brought about which resembles that previously observed in animals treated with cortisone, thorotrast, and trypan blue. An intadermal injection of toxin in these animals is followed by the appearance of hemorrhagic necrosis at the injected skin site similar to the local Shwartzman reaction. An intravenous injection of toxin is followed by the development of bilateral cortical necrosis of the kidneys, indistinguishable from the characteristic lesion of the generalized Schwartzman reaction. Moreover, in some of the animals treated with colloidal iron an intradermal injection of toxin was followed by death with renal cortical necrosis, indicating that some degree of absorption of toxin from the skin had occurred. In previous studies it was shown that such absorption does not take place in normal rabbits, but

is demonstrable in animals treated with cortisone or thorotrast(1,4).

It is essential that toxin be given after the injection of the colloidal materials. When the order is reversed and toxin injected beforehand, no lesions of the skin or kidneys occur.

The observations add support to the previously stated hypothesis(1,3,4) that the reticuloendothelial system is in some way concerned with defense against the necrotizing effects of gram-negative bacterial toxins, and interference with the normal functioning of this system results in a new kind of vulnerability to toxin. The possibility that a comparable interference with detoxification may be the function of the first or "preparing" injection of toxin, in the local and generalized Shwartzman reactions, is a subject for further investigation.

Summary. The intravenous injection of colloidal carbon or saccharate of iron oxide produces, in the rabbit, an alteration in the reactivity to gram negative bacterial toxins which resembles the effect of cortisone, thorotrast and trypan blue. An intradermal injection of toxin in such animals causes a local reaction of hemorrhagic necrosis which is similar to the local Schwartzman reaction, and an intravenous injection causes the development of bilateral cortical necrosis of the kidneys resembling the generalized Shwartzman reaction. It is suggested that the colloidal materials may produce these effects by interfering with normal protective functions of the reticuloendothelial system.

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Effects of d-Amphetamine on the Electroencephalogram of the Dog.* (20227)

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The central stimulating properties of sympathomimetic amines have been studied by observing the effects of these drugs on the spontaneous activity of rats. Schulte *et al.* (5) found that d-amphetamine produced the greatest increase in activity of 75 compounds studied. We felt that it might be of value to supplement such experiments on rats by observations on the electroencephalogram (EEG) of the dog. Although we have found no published reports on the EEG effects of d-amphetamine, studies have been made on the racemic form, *dl*-amphetamine. Gibbs and Maltby(2) found that this drug increased the frequency of the human EEG. In the rabbit, this drug potentiates the spread of a response from a stimulated area of the cortex to distant areas (Misrahy and Toman)(3).

The present paper describes an effect of *d*-amphetamine on the EEG of the dog. In the experiments described in Part I, dogs were prepared under thiopental anesthesia and then immobilized with decamethonium (C_{10}). This procedure permitted accurate recording of the EEG. To correlate the EEG changes produced by this drug with its effect on behavior, we used trained unanesthetized dogs, as described in Part II.

Part I—Thiopental—C_{10} Dogs. Procedure. The method is based on that described by Schallek and Smith(4). Dogs were prepared under thiopental anesthesia. Artificial respiration was instituted through a tracheal cannula, while hypodermic needles were inserted

* A brief report on this work was presented at the Chicago meeting of the Am. Physiol. Soc., April 9, 1953. Abstract appeared in *Fed. Proc.*, 1953, v12, 126.

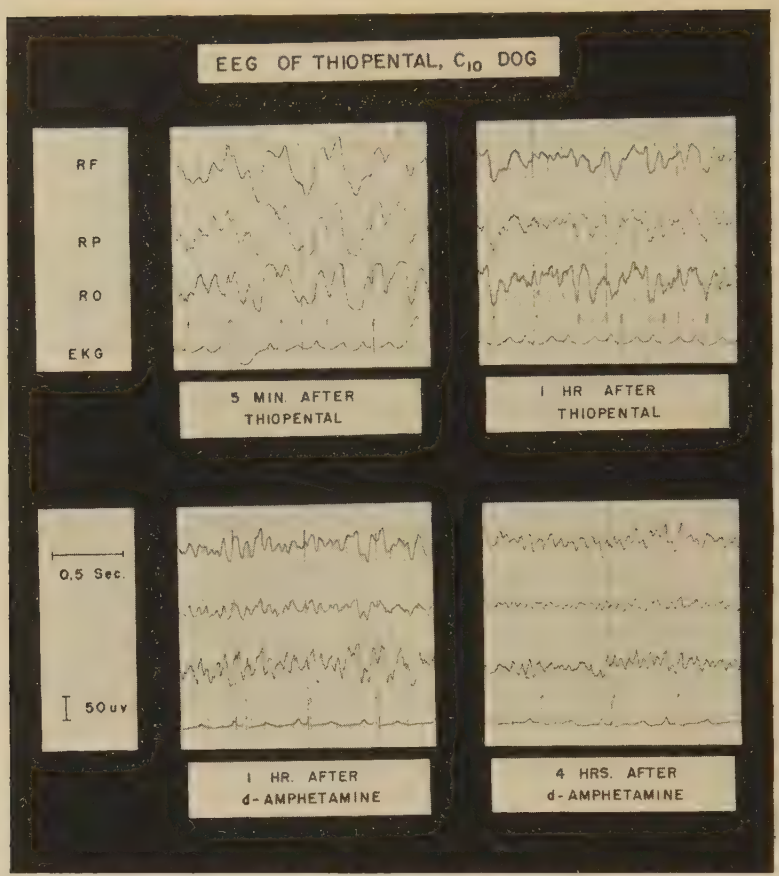

FIG. 1. EEG of thiopental-C_{10} dog. First 3 lines in each record are the EEG, right frontal, right parietal and right occipital; the fourth line is the EKG, Lead II. Paper speed, 6 cm/sec. Aug. 27, 1952—Dog No. 229, ♀—6.5 kg; Record A—5 min. after thiopental, 10 mg/kg, I.V.; Record B—55 min. after A; *d*-Amphetamine, 10 mg/kg, I.V., inj. immediately after B; Record C—one hr after B; Record D—4 hr after B.

in a femoral vein for injecting solutions and in the opposite femoral artery for recording blood pressure. The EEG was recorded from steel needles inserted in holes drilled through the skull; a Grass Model III C electroencephalograph was used. The electrocardiogram (Lead II) was recorded on the same machine. On completion of the operative procedure, the areas around the incisions were infiltrated with procaine and the animals immobilized with decamethonium. The drug being tested was injected one hour after the last injection of thiopental. Records were taken at periodic intervals for four or five hours. At the conclusion of the experiment, the animal was sacrificed. For analysis of the results, the dominant frequency and amplitude were measured on typical sections of the records.

Results. Preliminary experiments indicated that the effects of *d*-amphetamine are maximal at 5 and 10 mg/kg, I.V. The EEG changes consist of a progressive increase in frequency combined with a decrease in amplitude (Fig. 1).

Ten additional experiments performed with *d*-amphetamine and ten control experiments permitted further analysis of the results (Table I). The control animals were injected with 10 ml 0.9% NaCl. The progressive increase in the frequency of their EEG's seems to be correlated with recovery from the thiopental. Although the animals were stirring half an hour after injection of this anesthetic, the EEG suggests that traces of the agent remained long after injection. Brodie *et al.*(1) found that appreciable amounts of thiopental

TABLE I. Effects of d-Amphetamine on EEG of Thiopental-C_{10} Dogs.

	Multiples of initial EEG frequency (each figure is avg of 10 animals)						
	Time after injection						
	0 min.	15 min.	30 min.	1 hr	2 hr	3 hr	4 hr
Controls	1.00	1.03	1.09	1.19	1.59	1.69	2.03
d-Amphetamine, 10 mg/kg, I.V.	1.00	1.38	1.80*	2.18*	2.49	2.67	3.11
Ratio: d-Amphetamine/controls	1.00	1.34	1.65	1.83	1.56	1.58	1.53

* Denotes statistically significant difference when compared with control value ($P = .05$).

may be recovered from the dog twenty-four hours after administration.

The ratio of the *d*-amphetamine series to the controls indicates that the effects of this drug are maximal within the first hour after injection. This result accords with the observation in Part II that in unanesthetized dogs the peak action of *d*-amphetamine occurs within the first hour.

The question remains as to the nature of the EEG change produced by *d*-amphetamine. Is it merely some unimportant side-effect, or is it related to the central stimulation that is sought with this drug? To answer this question, we studied the effects of *d*-amphetamine on trained unanesthetized dogs, as described in Part II.

Part II—Unanesthetized Dogs. Methods. Dogs were trained to lie quietly on the table. Electrodes were attached to the shaved scalp with bentonite paste and then secured with collodion, using the method described in the "Instruction Manual for the Grass Electroencephalograph." The electrodes, made by the Grass Instrument Company for clinical use, are cup-shaped discs of pure silver, about 8 mm in diameter. Records were made of the following 4 types of behavior: *"Sleeping"*—the dog lies on the table completely relaxed, eyes tightly shut, paying no attention to ordinary laboratory sound. Records were not made until the animal had been in this state for several minutes. *"Dozing"*—the eyelids are lightly shut; the dog can be aroused by slight noises. *"Resting"*—the dog lies quietly with open eyes. *"Alert"*—the animal has just been aroused by the sound of a whistle or by tapping on the table. The signal had to be carefully graded to attract the dog's attention without causing movement, as the resulting muscle artifacts would obscure the EEG.

Drugs were injected intravenously. To avoid damage to the veins, only one injection was given to a dog on any one day; an interval of several days was allowed between injections. EEG records made following drug injection were compared with controls made on the same day before the drug was injected. Although it was easy to observe the effects of stimulant drugs on the behavior of unanesthetized dogs, it was more difficult to study the corresponding EEG changes. This is because doses of a drug producing sufficient central stimulation to change the EEG were apt to produce so much gross movement that the record was obscured by muscle artifacts.

Results. A. Untreated Dogs. As shown in Fig. 2, the EEG of a dog in deep sleep is composed of low frequency, high amplitude waves. As the dog is first gently awakened and then fully alerted, there is a rise in frequency, accompanied by a fall in amplitude. Comparison with Fig. 1 indicates that these changes are similar to those following the injection of *d*-amphetamine in thiopental-C_{10} dogs.

B. Drug Experiments. These experiments, carried out on 3 different dogs, may be summarized by the abridged protocols in Table II. It is evident that with *d*-amphetamine, in doses above 0.01 mg/kg, I.V., there is: (1) a progressive increase in motor activity. The maximum effect occurs within the first hour. (2) some evidence for a corresponding increase in the frequency of the EEG. This increase appears most regularly in the "resting" record.

Discussion. The dominant frequency of the EEG in unanesthetized dogs has been shown to increase during periods of sensory stimulation (Swank and Watson)(6). A rise in the frequency of the human EEG has been shown to follow the shift from "eyes closed" to "eyes open while reading"; a similar though smaller

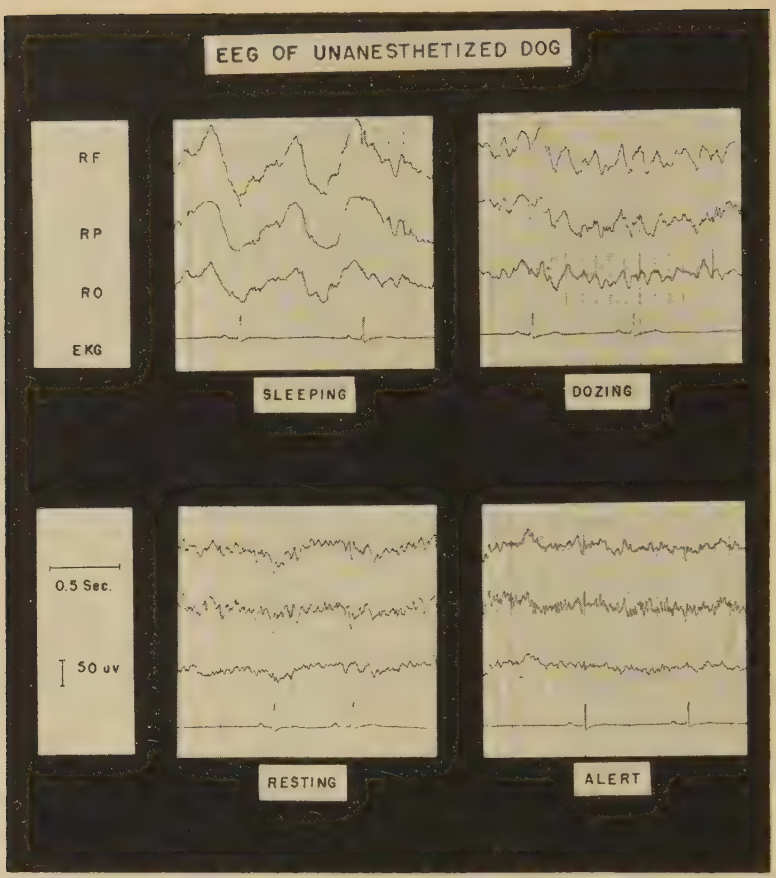

FIG. 2. EEG of unanesthetized dog. Arrangement as in Fig. 1. July 29, 1952—Dog No. 2, ♀—6.0 kg; Record A—sleeping; Record B—dozing; Record C—resting; Record D—alert.

shift follows the intravenous injection of 20 mg of *dl*-amphetamine (Gibbs and Maltby(2).

The present study on the dog agrees with this study on man in showing a parallel between the EEG effects of sensory stimulation and of *d*-amphetamine (compare Fig. 1 and 2). The evidence indicates that the effects of *d*-amphetamine on the EEG of the dog are parallel to its effects on behavior (Table II). The EEG changes introduced by *d*-amphetamine, therefore appear to be related to the central stimulation produced by this drug.

TABLE II. Effects of d-Amphetamine on Unanesthetized Dog.
Dog No. 1—♀, 6.3 kg.

Date, 1952	Dose of d-Amphetamine, mg/kg I.V.	Frequency of "resting" EEG			Effects on behavior
		Before inj.	After inj.	Change	
7/24	.0002	28/sec.	28/sec.	0	No change
7/28	.001	30	30	0	" "
7/31	.010	30	32	+2	Some movement after inj.
8/ 5	.013	24	28	+4	Slight restlessness after 30 min. No desire for sleep
7/18	.015	26	34	+8	Had to be comforted and later restrained
8/ 8	.020	30	42	+12	After 30 min., restlessness & increased body movement
7/17	.030	30	—	—	Too much movement to get any record

Summary. (1) In dogs prepared under thiopental and then immobilized with C_{10}, the injection of *d*-amphetamine causes a progressive increase in the frequency of the EEG, with a corresponding decrease in amplitude. These effects are maximal within the first hour. (2) When unanesthetized dogs are gently roused from sleep and then fully alerted, there is a rise in the frequency of the EEG, accompanied by a decrease in amplitude. (3) When *d*-amphetamine is injected into unanesthetized dogs, there is an increase in motor activity, the peak effect being reached within the first hour. There is some evidence for an increase in the frequency of the EEG. (4) These findings suggest that the effects of *d*-amphetamine on the EEG of the dog are parallel to its effect on behavior.

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Hormonal Influences on Liver Lipid Partition, Carbohydrate and Electrolyte Metabolism in the Rat. (20228)

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Among recent evidence indicating there exists an association between potassium and carbohydrate metabolism are the effects of insulin, epinephrine, and the infusion of large quantities of glucose upon the potassium level of plasma or serum. It was reported from this laboratory that following epinephrine and insulin injection in intact rats the plasma potassium level was lowered; this effect on potassium was also found after epinephrine in adrenalectomized or adrenal-demedullated rats but not after insulin(1-3). It was also reported that insulin and epinephrine induced divergent changes in the potassium content of liver and skeletal muscle(4-5). Further elucidation of the mechanism of action of insulin and epinephrine on the potassium content of plasma and tissues was desirable. Since it is increasingly evident that lipid and carbohydrate metabolism are interdependent phenomena, it seemed important to determine the effects of certain induced endocrine conditions upon liver lipid partition. In contrast to the present knowledge of the effects of hormones and their regulatory roles in carbohydrate and protein metabolism, information concerning hormonal influences upon lipid metabolism is limited. Experimental and clinical observations indicate that the endocrine secretions of the pancreas, thyroid, and adrenal cortex affect serum lipid partition(6-10). Evidence has been reviewed(11) which indicates that adrenal cortical homones either directly or through effects upon carbohydrate and protein metabolism have a critical influence upon lipid metabolism. Levin and Farber(12) reported increased total liver fat in the mouse following certain types of stress, but not after others. These investigators concluded that the stress per se did not initiate the "mobilization" of fat to the liver since it was prevented if the increased caloric requirements were met by the administration of glucose. However, Brownell, Hartman and Liu(13) reported experiments in the dog which indicated that there was a distinct hormone secreted by the

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adrenal cortex which influenced the deposition of liver lipids in test mice.

The interrelationships of insulin, epinephrine, and adrenocortical hormones in the regulation of various phases of carbohydrate metabolism are well known. Since the existence of an intimate relationship between carbohydrate and lipid metabolism is increasingly evident(14,15), experiments on the effects of insulin, epinephrine, intravenous glucose administration, and the influence of certain endocrine deficient conditions upon liver lipid partition were performed. At the same time determinations were also made of the liver and plasma water and electrolytes, liver glycogen, and plasma glucose content.

Materials and methods. Male Wistar rats weighing 250-300 g were used. A laboratory chow biscuit and tap water was given *ad libitum* and greens twice weekly. A 1% NaCl solution replaced the tap water for those groups of rats from which the adrenal glands were removed. The animal types used in the experiments reported here were: Intact: Adrenalectomized (5-6 days); and Adrenalectomized-Alloxan injected. In each of these categories there were control and treated groups as described in the experimental sections. *Adrenalectomy* was done by the dorsal-lumbar approach 5-6 days before the predicted time for obtaining tissues from these animals. In order to have groups of rats which were deficient in both adrenal gland secretions and also insulin, advantage was taken of the property of alloxan for selective destruction of pancreatic islet cells. Rats 2-3 days post-adrenalectomy were injected with Alloxan monohydrate (Eastman No. 1722) directly into an exposed saphenous vein at a dose level of 40 mg/kg body weight approximately 65 hours before sacrificing the animals. Epinephrine, insulin, and glucose were injected 60 minutes before tissue samples were to be removed from the designated groups, as follows: Epinephrine (Adrenaline tablets; Parke, Davis & Co.; prepared fresh for use in isotonic saline solution) was injected subcutaneously at a dose level of 0.04 mg/100 g body weight (approx. 0.2 ml); Insulin, (Iletin, regular; Lilly) was injected subcutaneously at a dose level of 0.5 Unit/rat (0.6 ml); the glucose (208 mg as an 8% solution freshly prepared in Sorenson's sodium phosphate buffer 15/M, pH 7.4) was injected slowly into an exposed saphenous vein. All surgical procedures, injections of agents, and the removal of tissues for analyses were done with the rats anesthetized with EVIPAL (n-methylcyclo-hexenyl - methyl barbituric acid). All animals in these experiments were fasted a minimum of 16 hours (overnight). At the designated time of the experiment, cardiac blood was obtained, immediately centrifuged, and aliquots of plasma taken for the determination of sodium, potassium, glucose and water content. The abdomen and chest were opened, the heart incised and allowed to bleed. A specimen of liver for glycogen determination was taken, gently blotted on filter paper, and put in a tared flask charged with 30% KOH. Another specimen of liver (approx. 1 g) was taken for the determinations of water and electrolytes content. The remainder of the liver was dissected free of adherent tissues and taken for the determination of liver lipids (total and fractions). All tissue samples were weighed on a chainomatic balance immediately after removal from *situ*.

The procedures for the determinations of plasma and tissue water and electrolytes were the same as previously described(2). Sodium and potassium were determined with the aid of a duo-optical designed internal lithium standard flame photometer; plasma glucose by the photometric method of Kingsley and Reinhold(16); liver glycogen by the method of Good, Kramer and Somgyi(17) and glucose equivalents after acid hydrolysis by iodometric titration method of Somogyi(18). The liver samples taken for lipid analyses were macerated in a mortar with sand and taken up in 95% ethyl alcohol. Extraction of the liver lipids was accomplished by refluxing 3 times with 95% alcohol and thrice with ether followed by distillation under reduced pressure of the combined extracts. The residue was taken up in petroleum ether (30-60 degrees); washed 3 times with an equal volume of water; then the petroleum ether extract was dried over anhydrous sodium sulfate and made up to a convenient volume. Suitable aliquots

TABLE I. Liver and Plasma Water and Electrolytes, Liver Glycogen, and Plasma Glucose Content in Intact, Adrenalectomized, and Adrenalectomized-Alloxan Groups of Rats.

	Liver constituents (per kg wet tissue)				Plasma constituents			
						K	Na	
Groups	Water, g	K, m.eq.	Na, m.eq.	Glycogen, mg %	Water, %	(m.eq./kg plasma water)		Glucose, mg %
Intact (12)	695±2	96.8±1.7	30.3± .7	20± 3	92.8±.1	5.36±.12	161.0±1.1	72±28
Adrenx (8)	*716±2*	94.0±1.2	28.8±1.2	25±12	93.7±.8	5.78±.19	159.4±2.0	*45± 2*
Adrenx-alloxan (8)	*707±2*	94.1±1.3	*34.8± .7*	*79±32*	92.8±.2	*7.69±.56*	165.4±3.0	75±15

Figures in parentheses are No. of animals in each group. All values are mean ± S.E.
Italicized values in table are significantly different from controls; "P" = <0.05.

of the petroleum ether were taken for the determination of the lipid partition as follows: Total lipids calculated from weight of residue after distillation of petroleum ether and drying to constant weight; lipid phosphorous by a modification of the Youngsburgs' method and the method of Fiske and SubbaRow(19); phospholipids (derived from the lipid phosphorous values x 26); total and ester cholesterol by the method of Schoenheimer and Sperry(20); neutral fat content was derived by calculation of difference.

Results. An established procedure in experiments to elucidate the effects of endocrine products is to study the effects of ablation of the secretory glands upon the biochemical constituents or physiological processes under investigation. Since phenomena observed following the removal of one type of endocrine gland may be the result of either the induced specific glandular deficiency or the unopposed action of the homonal products of another gland(s), it is often desirable in studies of intermediary metabolism to use animals with double glandular ablations. In this experiment the liver lipid partition, electrolytes and other constituents of liver and plasma were determined in intact, adrenalectomized, and adrenalectomized-alloxanized groups of rats.

Exp. I. The mean values of water and electrolytes content of liver and plasma together with the glycogen and glucose content of the respective tissues are presented in Table I. The means in each gland-deficient animal group were tested for statistically significant difference from the intact group. In the adrenalectomized group the liver water content was greater, and the plasma glucose level was significantly lower than the respective means of the intact group. These results are similar to those generally found in this type of animal maintained on a saline solution for drinking purposes. In the adrenalectomized-alloxanized group the means of liver water, sodium, glycogen, and plasma potassium content were found to be significantly greater than the respectives mean values in the intact group. The normo-glycemic level in these animals in association with an increased liver glycogen are noteworthy. Although no definite conclusions regarding the metabolic status in these animals can be made, the data of this group (examined against the results of the other two groups) suggests that the level of carbohydrate utilization was decreased, or that there was an increased formation of glycogen from intermediates of fat metabolism in the absence of insulin(21). The increased plasma K level probably reflects an inefficient utilization of glucose in this group.

In Fig. 1 are presented graphically the mean values of the liver lipid partition in intact, adrenalectomized, and adrenalectomized-alloxanized groups of rats. The means of the latter two groups were tested in turn for statistically significant difference from the respective mean values of the intact group. The influence of the altered hormonal conditions upon liver lipids composition is evident. The total lipid content in the two types of endocrine deficient rats was significantly lower than in the intact rats. Also, the mean total lipid content in the adrenalectomized group was significantly lower than that of the group with the dual glandular ablation. The phospholipid fractions were significantly lower, and the neutral fat fractions were significantly greater in the two

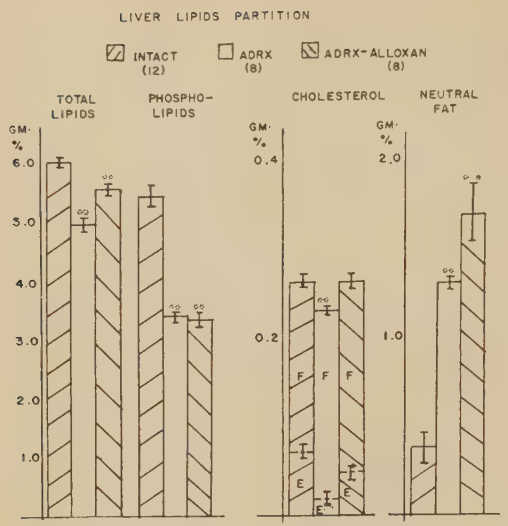

FIG. 1. Starred lipid values are significantly different from controls: *"P" = <0.05; **"P" = <0.01.

groups with endocrine deficiencies than the respective means found in the intact group. However, the neutral fat fraction in the adrenalectomized-alloxanized group was greater than that of the adrenalectomized group. The liver cholesterol fractions (total and ester) of the adrenalectomized animals were lower than the controls. It is noteworthy that the pattern of changes in the liver lipid partition in both the adrenalectomized and the dual-glandular deficient groups were the same except for the cholesterol fractions. These data suggest that the differences in liver lipid partition in the two endocrine deficient groups may be attributed essentially to the imposed condition of adrenal insufficiency and not to the consequence of an unopposed action of insulin. However, that the latter hormone did exert an influence upon liver lipid composition in the adrenalectomized group is indicated by the differences in mean values for total lipids, cholesterol, and neutral fat fractions in these two types of animals.

Exp. II. In this experiment observations were made on the liver lipid partition, electrolytes, and other constituents of liver and plasma in groups of intact rats not treated, and 60 minutes after epinephrine or insulin injection. It had previously been demonstrated that both of these hormones induced a significant decrease in the plasma potassium content of intact rats but divergent effects were found in the potassium content of plasma and other tissues in adrenalectomized and adrenal-enucleated rats following epinephrine and insulin injection(1,3,5). Since the mechanism of action of epinephrine and insulin on tissue potassium content is unsettled, it was decided to observe the concurrent effects of these two hormones upon liver lipid partition, glycogen, electrolytes, and plasma constituents. The 60 minute period after treatment was chosen because of the established evidence that maximum changes in glycemic level occur at this time following the injection of epinephrine or insulin. The mean-results of liver and plasma water, electrolytes, liver glycogen, and plasma glucose 60 minutes after the injection of each of these hormones in intact rats were compared with the respective mean levels in the untreated rats for statistically significant difference by Fisher's "t" test. The data are presented in Table II. The results are in agreement with data previously reported from this laboratory after similar treatment of intact rats; *viz,* a significant fall in plasma potassium, increased plasma sodium content, and the hyper-and hypo-glycemic levels consequent to epinephrine and insulin treatment, respectively. Inspection of these data shows that none of the other constituents of liver and plasma in the treated groups were significantly different from the respective mean values of the untreated intact group.

In Fig. 2 are presented the mean-results of the liver lipid determinations in the groups 60 minutes after epinephrine or insulin injection compared with the respective mean values of the untreated controls. Inspection of the data in Fig 2 shows that following the administration of either hormone the level of the total lipid in the liver was not different from the controls. However, the partition of liver lipids (phospholipids, cholesterol, and neutral fat fractions) were significantly changed in the groups injected with epinephrine or insulin. The greatly increased neutral fat fraction in the two treated groups is particularly noteworthy since this change evi-

TABLE II. Liver and Plasma Water and Electrolytes, Liver Glycogen, and Plasma Glucose Content in Groups of Intact Rats 60 Minutes after Epinephrine and after Insulin.

	Liver constituents (per kg wet tissue)				Plasma constituents			
Groups	Water, g	K, m.eq.	Na, m.eq.	Glycogen, mg %	Water, %	K (m.eq./kg plasma water)	Na (m.eq./kg plasma water)	Glucose, mg %
Not treat. (12)	695±2	96.8±1.7	30.3± .7	20± 3	92.8±.1	5.36±.12	161.0±1.1	72±28
Epinephrine (8)	697±2	97.3±1.3	28.7±1.0	14± 1	92.6±.1	*4.32±.19*	*166.7± .9*	*230±17*
Insulin (6)	698±3	95.2±2.0	30.3±1.6	14± 1	92.8±.2	*4.47±.23*	*166.9± .7*	*44± 5*

Figures in parentheses are No. of animals in each group. All values are mean ± S.E.
Italicized values in table are significantly different from controls; "P" = <0.05.
Epinephrine inj. subcut. at dose level of 0.04 mg/100 g body wt.
Insulin (regular) inj. subcut. at dose of 0.5 unit/rat.

dently was sufficiently great to account for the apparent unaltered total lipid content although the phospholipid fraction was significantly lower in both groups than in the untreated group.

Exp. III. In the 2 experiments presented above the effects of insulin and epinephrine injection, and the influence of certain deficient endocrine conditions upon liver lipid partition as well as other constituents of liver and plasma were determined. Since the hormones injected and the induced endocrine conditions in the groups of the preceding experiments are known to stimulate or adversely affect carbohydrate metabolism, it was decided to observe the effect of glucose administration on the liver lipid partition and the other constituents of liver and of plasma in comparable groups of animal preparations. In this experiment the liver and blood were obtained for the several biochemical determinations 60 minutes after the intravenous injection of 208 mg of glucose in intact, adrenalectomized, and adrenalectomized-alloxinized groups of rats. The mean-results found in these groups compared with the respective means found in similar groups of untreated rats are presented in Table III and Fig. 3. Inspection of the data in Table 3 shows that in the adrenalectomized group the plasma glucose level was significantly greater after the glucose infusion, and in the adrenalectomized-alloxanized group there was a marked hyperglycemia 60 minutes after glucose compared to the mean plasma glucose levels in the respective untreated groups. The liver glycogen content after glucose infusion in the intact and adrenalectomized groups was significantly greater than in their untreated controls; but the glycogen content was not altered in the group with dual-glandular deficiencies after the glucose infusion. The association of the marked hyperglycemia and the unchanged glycogen content in the latter group conform with the expectancy of such results in these animals in view of the classical defects known to exist in carbohydrate assimilation and utilization in the absence of both adrenocortical hormones and insulin. The only significant changes in plasma potassium and sodium concentration observed in these experiments was found in this group. The plasma changes may possibly be the resultant of the influences of a definite diuresis (consequent to the hyperglycemia) which was observed in these animals at the time they were sacrificed. The only

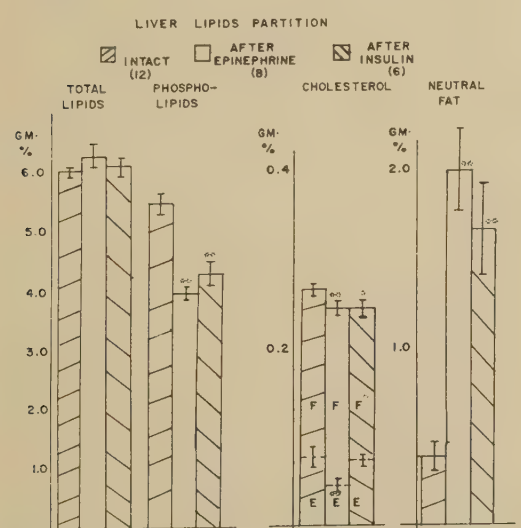

FIG. 2. Starred lipid values are significantly different from controls: * "P" = <0.05; ** "P" = <0.01.

TABLE III. Effect on Liver and Plasma Water and Electrolytes, Liver Glycogen, and Plasma Glucose Content 60 Minutes after Intravenous Glucose Administration in Intact, Adrenalectomized, and Adrenalectomized-Alloxan Groups of Rats.

	Liver constituents (per kg wet tissue)				Plasma constituents			
						K	Na	
Groups	Water, g	K, m.eq.	Na, m.eq.	Glycogen, mg %	Water, %	(m.eq./kg plasma water)		Glucose, mg %
Intact:								
Not treated (12)	695±2	96.8±1.7	30.3± .7	20± 3	92.8±.1	5.36±.12	161.0±1.1	72±28
Glucose, i.v. (12)	694±2	97.3±1.6	28.3± .6	*347±45*	*93.2±.1*	5.26±.18	165.8± .7	*111± 5*
Adrenx:								
Not treated (8)	716±2	94.0±1.2	28.8± .6	25±12	93.7±.8	5.78±.19	159.4±2.0	45± 2
Glucose, i.v. (8)	*728±3*	95.5±3.3	29.8±1.0	*110±29*	94.4±.2	5.27±.24	157.2±2.3	*109±12*
Adrenx-alloxan:								
Not treated (8)	707±2	94.1±1.3	34.8± .7	79±32	92.8±.2	7.69±.56	165.4±3.0	75±15
Glucose, i.v. (6)	*723±3*	93.3±1.9	*28.7±1.1*	113±21	*93.7±.1*	*5.52±.16*	*151.5±1.1*	*500—*

Figures in parentheses are No. of animals in each group. All values are mean ± S.E.
Italicized values in table are significantly different from controls: "P" = <0.05.
Glucose inj. intrav. (208 mg) 60 min. before tissues taken for analyses.

other notable changes in tissues constituents shown in the data of Table III were the small but statistically significant increased plasma water in the intact group and those with duo-endocrine glands deficiencies, and the increased liver water content in the latter and the adrenalectomized groups.

The liver lipid partition 60 minutes after intravenous glucose administration in intact, adrenalectomized, and adrenalectomized-alloxanized groups of rats compared with the respective mean values in their untreated controls are presented in Fig. 3. Total lipid content was unchanged in intact rats; but the phospholipids, total and ester cholesterol fractions were significantly decreased, and the neutral fat fraction was significantly greater after the glucose infusion. In the adrenalectomized group the only significant changes were increase in total liver lipids and in the neutral fat fraction. In the adrenalectomized-alloxanized group liver total lipids content and the neutral fat fraction were significantly lower, but the phospholipid fraction was significantly greater after glucose infusion than the respective mean values of their untreated controls.

Comment and summary. The experiments presented here represent an attempt to obtain data on the comparative influences of short-term hormonal overdosage or endocrine insufficiencies upon electrolyte, carbohydrate, and lipid metabolism followed concurrently in the animal. The major purpose of these studies was to secure information on the metabolic patterns of the two latter foodstuffs which would enable an evaluation of the endocrine influences upon potassium content and distribution in tissues. It is generally agreed that hormones modify the rates of metabolic reactions. It was our intent in Exp. I (Table I and Fig. 1) to obtain data concerning a number of constituents of liver and plasma in intact, adrenalectomized, and adrenalectomized-alloxanized which would allow a comparative evaluation of the metabolic status of the latter two types of animals with that of the intact rat. The two endocrine-deficient kinds of animals which were used in this comparative study were considered desirable for our purposes since it is well known that certain adrenocortical hormones and insulin have similar resultant influences upon certain phases of carbohydrate metabolism (glycogen formation) and are dissimilar in their resultant influences on other phases of carbohydrate metabolism (glucose oxidation). There is also increasing evidence that adrenocortical hormones and insulin are antagonistic in their resultant influences upon the mobilization of fatty acids to the liver and the mobilization of depot fat. It is apparent therefore that caution must be used in relating the level of tissue constituents to the effects of a specific hormone on intermediary metabolism or the absence of a gland in that the observed effects may be due to "unrestrained" influences

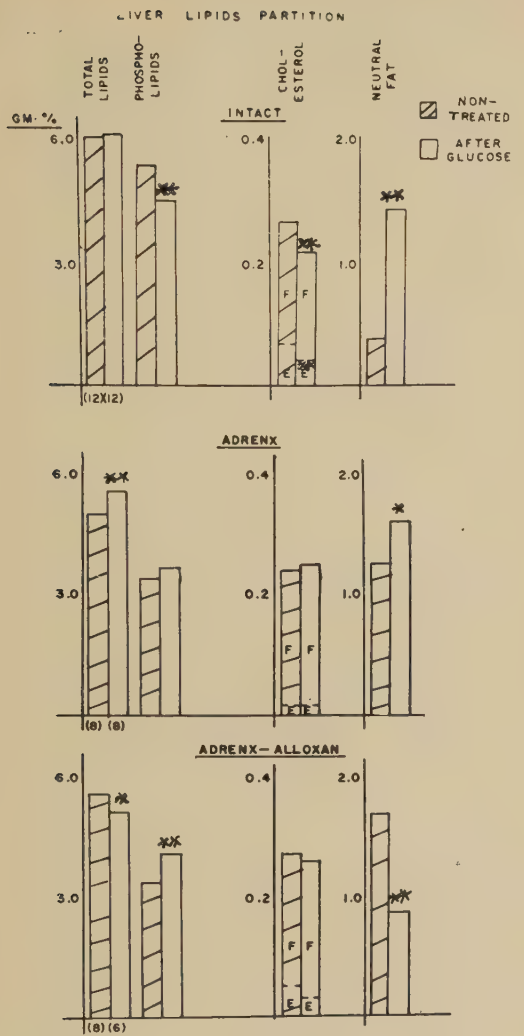

FIG. 3. Starred lipid values are significantly different from controls: * "P" = <0.05; ** "P" = <0.01.

of an antagonistic hormone(s). Examination of the data in Experiment I within this context suggests that in the adrenalectomized rat carbohydrate oxidation, and lipogenesis or neutral fat mobilization was greater than in the intact rat. As stated above, this might be interpreted as the resultant influence of lack of adrenocortical hormones or the unopposed activity of insulin. Parallel inspection of the data of the adrenalectomized-alloxanized group suggests that glucose oxidation in the adrenalectomized group was proceeding under the unopposed action of insulin since in the former group the evidence suggests an amelioration of this metabolic status during the condition of dual-glandular insufficiencies (adrenal and insulin). However, it is equally evident that the status of the metabolism of fat as evidenced by the levels of neutral fat and phospholipid in the liver was not altered in the absence of insulin and adrenal hormones from that found in the absence of adrenal gland secretions alone. Thus the changes in the levels of the various lipids in the two groups from those found in the controls of Experiment I must be due mainly to the absence of the hormonal influences of the adrenal gland. A more detailed interpretation of the liver lipid changes must necessarily be tentative in view of the uncertainties regarding the metabolic interrelationships of the lipid fractions in the liver. It has been reported that in the depancreatized dog there is a marked increase in the turnover of liver phospholipids when exogenous insulin is withdrawn(22). Since the liver phospholipids probably are not involved in fat transport(23), the increased turnover suggested that some of the liver phospholipids participate in fat catabolism. In the present experiments the decrease in liver phospholipids observed in the animals with glandular deficiencies suggests an elevated level of fat metabolism. The increase in neutral fat could be due either to mobilization of fat from depots or an increased rate of synthesis in response to the elevated level of fat metabolism.

Information concerning the effect of epinephrine on lipid metabolism is very meagre. Cori and Cori(24) deduced from their data that fat is the fuel for the increased metabolism due to epinephrine. An increase in liver phospholipids and fatty acids 2-3 hours after subcutaneous injection of epinephrine in rabbits was reported by Pollack(25). There is increasing evidence however that insulin affects several phases of lipid metabolism. A marked reduction in the phospholipids of blood and liver in depancreatized dogs maintained with insulin was reported(26,27). A depression in lipogenesis in the alloxan-diabetic rat(28) and an increase in hepatic lipogenesis in the normal rabbit fed a high carbohydrate diet given insulin injection has been

reported(29). In general the evidence reported in the literature indicates that insulin's influence upon lipid metabolism is related through the effects of this hormone on glucose utilization. The data presented in Experiment II (Fig. 2) show that significant changes in the fractions of liver lipids followed the injection of insulin and epinephrine. However, the present data does not permit a definitive interpretation of a specific effect of either epinephrine or insulin on these lipid fractions because of the evidence recently reported that in the intact animal each of these hormones stimulates the secretion of the other hormone (30-32); the known effects which both of these hormones exert upon carbohydrate utilization and oxidation; and the increasing evidence of an influence of the latter processes upon lipid metabolism. A particularly puzzling aspect of the findings in this experiment is that the shift in the liver lipid pattern from that in the intact untreated animal is essentially the same found in the adrenalectomized, the adrenalectomized-alloxanized rats, and the intact rat which received injections of epinephrine or insulin. It would appear that the same changes in fat metabolism in the liver occurred in all 4 types of experimental animals. Further investigation of the effects of insulin and epinephrine with the purpose of delimiting their specific actions on liver lipid metabolism is presented in the succeeding paper.

The data of Fig 3 show clearly that administration of intravenous glucose affected the level of the liver lipid fractions. Also the results show that the endocrine status of the animals exerted a definite influence on the effects of the glucose administration upon the liver fractions. In the adrenalectomized group the increase in total lipid content was accounted for by the increased neutral fat fraction alone. In the intact and the adrenalectomized-alloxanized groups the changes in the neutral fat and the phospholipid fractions were in opposite directions following the glucose infusions. It is noteworthy that the liver glycogen content of the intact and adrenalectomized rats was significantly increased after the glucose (Table III) and in both these groups the neutral fat content was increased. The evidence presented is suggestive that in the absence of insulin and adrenal gland secretions there was a decrease in the level of fat metabolism following the glucose infusion. Furthermore, the data presented in Table III is suggestive that carbohydrate utilization was apparently proceeding at an extremely slow rate in the adrenalectomized-alloxanized group.

There are several general aspects of the liver lipid data obtained in these experiments which deserve special comment. First, significant changes in the level of the lipid constituents can occur with much greater rapidity (30-60 minutes) than is generally recognized. Thus, it appears that fat metabolism is as responsive to the changing metabolic requirements of the organism as is carbohydrate metabolism. Secondly, the changes in the levels of phospholipid and neutral fat were always in opposite directions. When changes occurred in the cholesterol fractions, they paralleled those for the phospholipids. These interrelationships are probably a reflection of the functions of these constituents in fat metabolism. Neutral fat being the form in which fat is mobilized and transported, and the phospholipid and cholesterol esters constituents involved in some stages of fat catabolism in the liver. Thirdly, it is apparent from the present data that the level of total fat in the liver is not a dependable criterion in studies on the influence of hormone deficiency or excess on fat metabolism in the liver. Some of the shifts in lipid pattern reported here occurred without any change being evident in total lipid values. The total lipid values found in the various experimental groups reported here appeared to be the resultant of opposing changes in the different fractions, and whether the values were the same, greater, or less than the respective controls was due to the quantitative rather than the qualitative nature of the changes in the level of the lipid constituents. Finally, it should be emphasized that the level of the various lipids found in these experiments are all within the so-called normal range for the rat. Thus, the significant changes are not due to grossly abnormal conditions but rather represent "normal" shifts in the pathways of metabolism in response

to changes in the hormonal pattern of the organism.

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Epinephrine and Insulin Effect on Potassium Mobilization: Relationship of Lipid and Carbohydrate Metabolism. (20229)

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The comparative effects of epinephrine and insulin on the potassium content of plasma, skeletal muscle, and liver in adrenalectomized-alloxanized rats given a glucose infusion were reported in a recent paper by Dury(1). The use of that type of animal and the design of the experiments were discussed in that report. The results indicated that the effects of epinephrine and of insulin upon tissue potassium content were of such divergent pattern that it reflected differences in the action of these two hormones on intermediary metabolism. Experiments were designed to obtain more detailed information concerning the constituents of the tissues of adrenalectomized-alloxanized rats given an infusion of a

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TABLE I. Effect of Epinephrine and Insulin Pretreatment of Adrenalectomized-Alloxan Groups of Rats Given Glucose Infusion upon Liver and Plasma Water and Electrolytes, Liver Glycogen, and Plasma Glucose Content.

	Liver constituents (per kg wet tissue)				Plasma constituents			
Groups	Water, g	K, m.eq.	Na, m.eq.	Glycogen, mg %	Water, %	K (m.eq./kg plasma water)	Na (m.eq./kg plasma water)	Glucose, mg %
Adrenx-alloxan: glucose, i.v.* (6)	723±3	93.3±1.9	28.7±1.1	113±21	93.7±.1	5.52±.16	151.5±1.1	500—
Glucose, i.v.+ epinephrine preRx (7)	717±3	*102.5±2.6*	30.0± .7	*257±15*	*92.8±.2*	4.96±.26	*163.3±3.0*	500—
Glucose, i.v.+ insulin preRx (5)	732±4	*102.1±1.9*	28.9±1.2	*585±161*	*92.9±.1*	*4.66±.15*	*160.6±1.4*	*270±20*

Figures in parentheses are No. of animals in each group. All values are mean ± S.E.
Italicized values in table are significantly different from controls; "P" = <0.05.
* Glucose (208 mg) inj. intrav. 60 min. before tissues taken for analyses.
Epinephrine (0.04 mg/100 g rat) inj. subcut. 30 min. after the glucose administered.
Insulin (regular, 0.5 unit/rat) inj. subcut. 30 min. after the glucose administered.

standard amount of glucose and pretreated with epinephrine or insulin. It is the purpose of this paper to report the comparative effects of these procedures upon liver lipid partition and glycogen content, plasma glucose, and the water and electrolytes content of liver and plasma.

Materials and methods. The materials and methods employed in the experiments reported here were identical with those described in the preceding paper(2). In order to have rats with combined adrenal and insulin insufficiencies available for these studies, rats 2-3 days after adrenalectomy were injected with a 5% solution of Alloxan monohydrate (Eastman No. 1722) directly into an exposed saphenous vein at the dose level of 40 mg/kg body weight. The rats were maintained on a 1% NaCl solution for drinking purposes and a chow biscuit *ad libitum* during the entire period after adrenal ablation. Tissues for analyses of liver and plasma constituents were taken approximately 65 hours after the alloxan injection (viz, 5-6 days after adrenalectomy). The following groups were used: (A) 60 minutes after intravenous administration of 208 mg of glucose as an 8% solution freshly prepared in Sorenson's sodium phosphate buffer M/15, pH 7.4. (B) 30 minutes after the infusion of the glucose solution, epinephrine (Adrenalin tablets; Parke, Davis & Co.) freshly prepared in isotonic saline was injected subcutaneously at the dose level of 0.04 mg/100 g body weight. Tissues for analyses were taken 60 minutes after the glucose administration (or 30 minutes after the hormone was injected). (C) 30 minutes after the glucose infusion, insulin (ILETIN, regular; Lilly) freshly prepared in isotonic saline was injected subcutaneously at the dose level of 0.5 Units/rat. Tissues for analyses were taken 60 minutes after glucose administration (or 30 minutes after the insulin injection). In all groups, cardiac blood was obtained by direct puncture, immediately centrifuged, and aliquots of plasma taken for determinations of water, electrolytes, and glucose content. Specimens of liver were taken for determinations of liver lipid partition, glycogen, water, and electrolytes content. All surgical procedures, and the injections of the various agents were done with the animals anesthetized with n-methylcyclo-hexenyl-methyl barbituric acid (Evipal).

Results. The means (±S. E.) of the plasma and liver constituents (other than the lipids partition) are presented in Table I. A statistically significant difference between the means of the respective values in each of the pretreated groups and those of the non-pretreated group were found for the following: In the epinephrine pretreated group the liver potassium and glycogen content, and the plasma sodium content were significantly greater than those of the controls; and the plasma water content was significantly lower than the con-

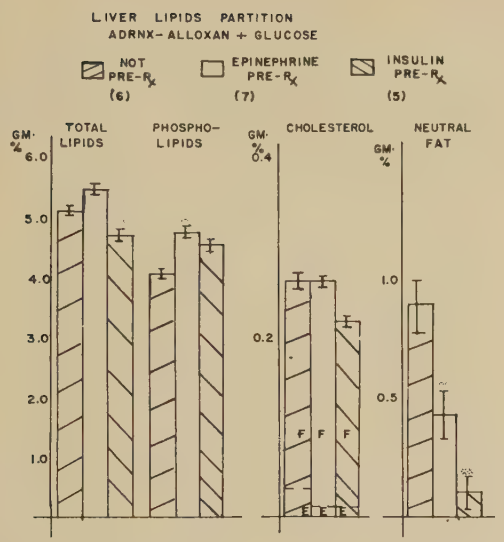

FIG. 1. Starred lipid values are significantly different from controls: *"P"= <0.05; **"P"= <0.01.

trol value. The high glycemic level is noteworthy in conjunction with the evidence of glucose utilization indicated by the increased liver glycogen content. The plasma potassium level although considerably lower than that of the control was found to be just beyond the 5% limit of significance. In the insulin pretreated group the liver potassium and glycogen content, and the plasma sodium content were significantly greater than those of the respective control values; and plasma water, potassium, and glucose content were significantly lower than the respective values of the controls. The change in glycemic level and the increased glycogen content of the liver are in accord with the known pattern of the influence of insulin action on carbohydrate metabolism. The lowered plasma potassium level consequent to insulin administration has been reported in man and animals several times [see references quoted by Dury(3)].

The partition of the liver lipids in these same three groups are illustrated in Fig. 1. In both pretreated groups the phospholipid fractions were significantly increased and the neutral fat fractions were significantly lower than those of the controls. Although the pattern of changes in the phospholipid and neutral fat fractions were similar in the two pretreated groups compared with the controls, it is evident that the extent of the drop in neutral fat in conjunction with the lowered total cholesterol fraction in the insulin pretreated group accounted for the significantly lower total lipid content in this group compared with the controls. The total lipid level in the insulin pretreated group was also significantly lower than the corresponding value in the epinephrine pretreated group.

Discussion. In the preceding paper(2) it was shown that there were distinct differences in the concentration of liver glycogen, plasma glucose and plasma potassium, and the partition of liver lipids in intact and adrenalectomized-alloxanized groups of rats given an infusion of glucose compared with their respective untreated controls. In the former group the results were generally consonant with the interpretation that the changes in the levels of the constituents reflected the predominant effects of insulin action in response to an imposed glucose load in the organism, viz, upon carbohydrate metabolism and (direct or indirect) effects upon lipid metabolism. However, in the rats with imposed dual-glandular deficiencies similarly treated with glucose there was no evidence of increased carbohydrate utilization; and the liver lipid partition was suggestive of decreased lipogenesis or decreased lipid mobilization. As was pointed out in the preceding paper(2) these divergent results after similar treatment in the two types of animal preparations did not warrant the deduction that these differences were due to the influence of insulin, per se, in the intact animals, or insulin lack in the adrenalectomized-alloxanized groups. The intimate interrelationships extant in the intact animal between insulin, epinephrine, adrenocortical steroids and carbohydrate utilization (particularly the elicitation of secretion of these hormones by any one of the others) were recently reported from this laboratory(3-5), by Somogyi(6), and by Euler and Luft(7). That the partition of liver lipids may likewise reflect the resultant of these hormonal interrelationships in the intact animal, especially after insulin or epinephrine injection, was indicated in the results and the discussion of Experiment II of the preceding paper(2).

The data of these experiments indicate that

a lowered plasma potassium level in the insulin pretreated group occured in conjunction with mobilization of potassium and glycogen into the liver. This, and the fall in glycemic level suggest that the alterations in plasma and liver potassium levels were induced in these animals consequent to insulin action promoting carbohydrate utilization. In accord with this, the fall in the level of liver neutral fat and the increase in the phospholipid fraction would follow due to a shift in the metabolic pathways of predominantly fat catabolism to a more normal pattern consequent to glucose and insulin availability. It is perhaps significant that the levels of the liver lipid fractions in these adrenalectomized-alloxanized rats pretreated with insulin closely resembled the values found (see previous paper) in the intact post-absorptive rats. It is evident that there were several changes in tissue constituents induced with insulin pretreatment all of which are in agreement with the generally accepted manifestations of insulin action upon metabolism.

The association of increased potassium and glycogen levels in the liver after epinephrine pretreatment suggests that the effect of the hormone on potassium mobilization was comparable to that of insulin. Likewise, the changes in the liver lipid partition in the epinephrine pretreated group indicate comparable effects upon fat metabolism as with insulin pretreatment, except for the extent of change in the neutral fat fraction. However, the high glycemic level and the considerably less liver glycogen content in the epinephrine pretreated group (in contrast to those found after insulin) indicate that the changes in liver and plasma potassium content cannot be attributed to an increased level of carbohydrate utilization in the epinephrine pretreated group. These findings are sufficiently notable to suggest that mobilization of potassium to the liver and the fall in plasma potassium level although the common resultant of epinephrine and of insulin pretreatment in these animals probably were not produced by the same mechanism of action with both these hormones.

Summary. The effects of epinephrine and of insulin pretreatment on liver lipid partition, glycogen, water and electrolytes content, and certain plasma constituents were compared in adrenalectomized-alloxanized groups of rats given a glucose infusion. A similar pattern of changes in liver lipid partition was found in both pretreated groups compared to the non-pretreated controls. An increased liver potassium and lowered plasma potassium content was found in both pretreated groups. However, differences in liver glycogen content and glycemic level in the pretreated groups were sufficiently notable to suggest that common resultant effects on potassium mobilization probably were not produced by the same mechanism of action of both hormones.

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Occurrence of Tumors in Agent-Free Strain $C3H_f$ Male Mice Implanted with Estrogen-Cholesterol Pellets. (20230)

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A line of agent-free strain C3H mice was originated in this laboratory in 1945, from a litter of high-mammary-tumor strain C3H mice taken from their mother by cesarean section and foster-nursed upon a low-mammary-tumor strain C57BL female without the mammary tumor agent. The line is now designated as strain $C3H_f$ (formerly $C3H_b$). In contrast with the low incidence of mammary gland tumors that was originally expected in this strain, mammary tumors appeared in 38% of the breeding females although these appeared at the advanced average age of 20 months and the incidence in virgin females was only 2% (1,2). Cell-free filtrates of these tumors failed to give a positive test for the agent, and, furthermore, there was no evidence of segregation of high-tumor lines in the pedigree chart as would have been expected had the agent been present in some of the females. In addition, studies of reciprocal crosses between strain $C3H_f$ and strain C57BL have failed to give evidence of any maternal influence in the development of the tumors in the $C3H_f$ females (2).

In view of the occurrence of mammary tumors in the breeding females of this agent-free strain, it seemed desirable to ascertain whether mammary tumors would occur in males of the strain treated with estrogen, and, if so, to test these tumors for the presence of the agent. This paper reports that mammary tumors did occur in such males and that tests of cell-free filtrates of these tumors gave no evidence of the presence of the agent. The incidence of hepatomas in these estrogen-treated $C3H_f$ males is also recorded.

Procedure. Sixty-eight strain $C3H_f$ males of litters from matings distributed at random throughout the pedigree chart were set aside for this study at the time they were weaned. When they were from one to two months of age a 5 to 8 mg pellet of cholesterol containing 10% of diethylstilbestrol was implanted subcutaneously into the right axilla of each. They were then set aside to be observed regularly for the appearance of tumors. Throughout the study they were kept in plastic cages with 8 males to the cage and were given an unlimited supply of Derwood pelleted food and tap water. Each animal was autopsied following the appearance of a tumor or when it was found to be moribund or was found dead in the cage. The tumors were fixed in Fekete's modification of Tellyesniczky's fluid (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), sectioned and stained with hematoxylin and eosin for histologic examination.

Results. A total of 60 males are included in the final tabulation. The seven that were deleted included 2 that died approximately one month after implantation of the pellet, one that was killed at 3 months of age and its organs used in another study, and 4 for which satisfactory records were not obtained since the animals died in the cages and were eaten by their cage mates. Tumors occurring in the 60 males are listed in Table I.

It is significant that mammary tumors occurred in 12 of these males. This incidence of 20% is not as high as the 38% incidence of mammary tumors observed in the breeding $C3H_f$ females(1), but it is significantly higher than the 2% incidence observed in $C3H_f$ females kept as virgins(2). The mammary tumors appeared in these males at from 9 to 22 months of age with an average age of 15.1 months compared with an average tumor age of 20.3 months reported in the $C3H_f$ breeding females. Histologically these 12 tumors were adenocarcinomas, 11 of which were classified as Dunn's type A, and one as Dunn's type B (3). None showed squamous metaplasia with keratinization or other unusual histologic structure such as has been observed in some of

* National Institutes of Health, U. S. Public Health Service.

TABLE I. Tumors Occurring in 60 Strain C3H$_f$ Male Mice Bearing Pellets of Diethylstilbestrol in Cholesterol and Living to an Average Age of 18.9 Months.

Type of tumor	No. mice with tumor	% with tumor	Avg tumor age, mo
Mammary tumor	12	20	15.1
Hepatoma	14	23.3	22.6
Pulmonary tumor	5	8.3	19.4
Subcutaneous sarcoma	1	1.7	24
Tumor of Harderian gland	1	1.7	20

the mammary tumors that arose in C3H$_f$ females spontaneously and following x-irradiation(4), and also observed by others (5-8) in mammary tumors that arose spontaneously or following treatment with a carcinogen in females of other agent-free lines.

While it is remarkable that 20% of these diethylstilbestrol-treated agent-free C3H$_f$ males did develop mammary tumors, this incidence is not as high as Shimkin and Wyman(9) observed in similarly treated C3H males of Andervont's subline with the mammary tumor agent. They reported an incidence as high as 86% in those implanted with pellets containing .6 mg diethylstilbestrol, a dosage comparable with that used in the present studies. Comparison between these incidences is probably justified since our subline was derived from Andervont's subline in 1941.

To compare with these treated C3H$_f$ males, 20% of which developed mammary tumors, 241 untreated C3H$_f$ males of the breeding colony have died or been killed and examined at an average age of 12.5 months and none has had a mammary tumor. Furthermore, mammary tumors have never been observed in males of the strain C3H breeding colony, and have been reported as occurring only very rarely in untreated male mice in other laboratories.

From a review of the literature it appears that the present observation is the first observation of the development of mammary tumors in estrogen-treated males without the agent. In Shimkin's review(10) a number of groups of male mice including strain 17 n.c., 19 x R_3 hybrids, strain C57BL, strain Ax, C57BL x CBA hybrids, strain C, and strain CBA, all of which did not carry the milk agent, were listed as having been treated with estrogen, and in no group did mammary tumors appear. It should be emphasized, however, that undoubtedly the C3H$_f$ mice have a more potent genetic influence for the development of mammary gland tumors than any of these other strains or hybrids.

Hepatomas occurred in 14 of the 60 C3H$_f$ males bearing estrogen pellets. The average tumor age was 22.6 months. In a group of 39 untreated C3H$_f$ males that lived to an average age comparable with that of these treated males, and that were controls in a concurrent study, hepatomas occurred in 18 at an average tumor age of 20.2 months(14). Comparison of these two groups indicates that the treatment with diethylstilbestrol reduced the occurrence of hepatomas for there is a significant difference between the two incidences; $X^2 = 5.63$; P is between .01 and .02. Andervont (12) observed incidences of hepatomas in groups of untreated C3H males ranging from 12 to 55%. When his groups are combined the incidence in the total of 409 males is 32.76% which is greater than that of the diethylstilbestrol-treated C3H$_f$ males reported here. He also noted an incidence in untreated C3H males without the agent that was higher than that of these treated C3H$_f$ males. A decrease in incidence of hepatomas was noted in castrated C3H males with the agent and in those without the agent.

The treatment with diethylstilbestrol did not, however, lower the incidence of hepatomas in the C3H$_f$ males to that of untreated C3H$_f$ females. In 188 C3H$_f$ breeding females that lived to an average age of 19.1 months, hepatomas occurred in only 9, and these occurred at an average age of 21.3 months(1). Furthermore, in a group of 100 strain C3H$_f$ virgin females that lived to an average age of 19.9 months, hepatomas occurred in 17 and at an average age of 23.8 months(2). Agnew and Gardner(13) noted a more striking reduction in incidence of hepatomas than that reported here when they injected estrogens into C3H male mice. The only hepatomas they observed in the treated males occurred in those that received very small doses of estrogen (1 μg or 3.3 μg per week). No hepatomas were observed in males that had received 8.3

TABLE II. Tests for Presence of Mammary Tumor Agent in Tumors that Arose in Treated C3H$_f$ Male Mice and in Breeding C3H Female Mice.

Tumor filtrate No.	Age tumor arose, mo	Test C3H$_f$ females: Number	Avg age, mo	No. with tumor	Avg tumor age, mo
		From treated C3H$_f$ ♂			
149	10	5	18.4	0	—
154	12	5	15.4	0	—
155	12	5	19.6	0	—
157	14	4	11.8	0	—
164	22	4	22	0	—
		From breeding C3H ♀			
150	7	5	17.6	2	16.5
151	*	4	14.8	3	14.7
152	*	4	10	1	9
153	7	5	14.8	2	19
156	10	4	17.3	1	14
158	7	5	22.8	4	21
161	9	4	16.3	3	17
162	13	4	16	4	16
163	9	4	21	1	11

* Unknown.

μg or more per week. Previous observations on the effect of estrogens on the incidence of hepatomas in male mice are reviewed by Agnew and Gardner(13).

Other tumors observed in the diethylstilbestrol-treated C3H$_f$ males included pulmonary tumors in 5 males, a subcutaneous sarcoma in one and a tumor of the Harderian gland in one. This incidence of pulmonary tumors is comparable with incidences recorded for untreated C3H$_f$ males and females(1,2,11).

Tests for mammary tumor agent. Five of the mammary tumors that arose in the treated C3H$_f$ males and that were selected at random were tested for the presence of the mammary tumor agent. Five percent extracts of each tumor were prepared in distilled water with a Waring blendor; cleared in a centrifuge at 1800 to 2000 RPM for 10 minutes; and filtered through a Selas micro-porous porcelain filter (porosity 03, maximum pore size .6 μ). To test for the agent .2 cc of the filtrate was injected intraperitoneally into 4 or 5 C3H$_f$ females 21 to 28 days of age. The test females were individually identified and observed throughout their life span for the development of mammary tumors.

Results of the tests of these C3H$_f$ tumors are given in Table II along with results of identical tests concurrently made of mammary tumors from breeding C3H females that have the agent. No mammary tumors appeared in any of the 23 females used to test the tumors from the treated C3H$_f$ males. In contrast, mammary tumors occurred in 21 of the 39 females used to test the 9 tumors from the C3H females, and none of the 9 filtrates failed to produce at least one tumor in the 4 or 5 test females. These tests, thus failed to give any evidence of the mammary tumor agent in the tumors of the C3H$_f$ males just as similar tests previously failed to give evidence of the agent in the mammary tumors that arose in the breeding females of this strain. Therefore, although mammary tumors arose in 20% of these diethylstilbestrol-treated C3H$_f$ males, they apparently arose in the absence of the agent.

Summary. 1. Mammary tumors occurred in 12 or 20% of 60 agent-free strain C3H$_f$ male mice implanted with pellets of cholesterol containing 10% of diethylstilbestrol. The average age at which the tumor arose was 15.1 months. Tests of cell-free filtrates of 5 of these tumors selected at random failed to give evidence of the presence of the mammary tumor agent. 2. Hepatomas occurred in 14 or 23% of the diethylstilbestrol-treated males at an average age of 22.6 months. This incidence is significantly less than that recorded for untreated strain C3H$_f$ males of a comparable age, but greater than that recorded

for untreated $C3H_f$ females of comparable age.

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An Aldoheptose Phosphate in a Polysaccharide Isolated from *Shigella flexneri.* (20231)

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The polysaccharide of the somatic antigen of *Shigella flexneri,* type 3, has been shown to consist mainly of D-glucose, L-rhamnose and D-glucosamine(1-3). Approximately 1% of the dry weight of the polysaccharide preparation was found to consist of non-dialyzable, acid-stable, organically bound phosphorus. After acid hydrolysis of the polysaccharide and isolation of the water soluble barium salts of the phosphate esters, the latter were found to contain some hexose-6-phosphate and a heptose phosphate(1,3).

Materials. The degraded polysaccharide corresponding to fraction 3 or 4 was used as a source of the phosphate esters(3). Dialyzed human seminal plasma was used as a source of acid phosphatase. Amberlite IR-120 cation exchange resin was used after charging with HCl and IR-4B anion exchange resin was charged with Na_2CO_3. We are indebted to Dr. N. K. Richtmyer at the National Institutes of Health for generous gifts of several heptoses.

General methods. Phosphorus was determined by the method of Fiske and Subbarow (4). Heptose determinations were made by the sulfuric acid-cysteine procedure (CyR) described by Dische for other sugars(5).* We have found it possible to characterize all 7-carbon sugars which have been made available to us by comparing spectra of each sugar after CyR4 and CyR10 reactions (*i.e.*, after heating for 4 and 10 minutes, respectively). The ketoheptoses produce spectra which differ from each other only slightly, but have a much greater absorption at 510 mμ than do the aldoheptoses. The latter produce spectra which are similar in the visible region, but vary in their ultraviolet portions. However, aldoheptoses having mirror-image configurations about the first five carbon atoms produce very similar CyR spectra. Results with the CyR10 reaction are shown in Fig. 1. B and C which have enantiomorphic configurations about the first 5 carbons have similar spectra. In like manner, the pair F and G show a marked resemblance in the ultraviolet

* Dr. Dische suggested in April, 1952 that the pink color having a maximum absorption at about 510 mμ which we obtained with our phosphate ester fraction in his CyR10 reaction might be due to the presence of a 7-carbon sugar. A paper by Dr. Dische on color reactions of heptoses is in press for the *J. Biol. Chem.*

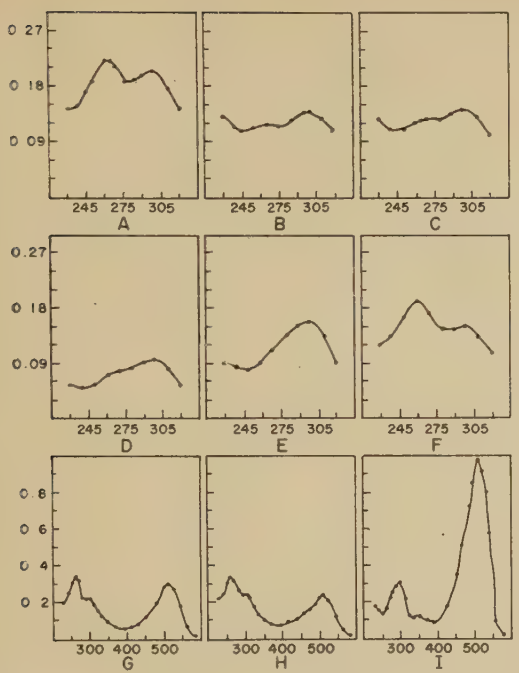

FIG. 1. Comparison of CyR10 spectra of several heptoses. In each case approximately 50 μg of sugar were present in 5.6 ml. The spectra were measured with a 1 cm light path in a Beckman spectrophotometer about 4 hr after the addition of cysteine. A, D-gluco-D-guloheptose; B, D-manno-D-gala-heptose; C, D-gulo-L-galaheptose; D, D-altro-D-glucoheptose; E, D-gala-L-glucoheptose; F, D-altro-D-mannoheptose; G, D-gala-L-manno-heptose; H, heptose from phosphate fraction of polysaccharide hydrolysate; I, sedoheptulosan. Ordinates: optical density, abscissae: wavelength in mμ.

region. The results with D and E are not as striking. Since no heptoses of the L-series were available, it is not certain whether the same results would be obtained with them. Hexoses corresponding to these pairs do not give ultraviolet spectra similar to those of the heptoses. If the complete spectra for the CyR4 and CyR10 reactions of all these sugars had been reproduced here, it would be seen that even those which have similar ultraviolet spectra differ in some characteristic manner such as whether or not the CyR4 and CyR10 spectra intersect at any point or to what extent the maxima change in intensity when the sugars are heated with acid for 10 minutes instead of 4 minutes. The *altro-sugars* were supplied as their hexaacetates. Since the acetyl groups are hydrolyzed during the heating with strong sulfuric acid in the CyR procedure, the spectra represent those which would be obtained with the free sugars. Sedoheptulosan (Fig. 1, I) is representative of the ketoheptoses. It may be seen that the spectrum for the heptose obtained from the phosphate fraction of the hydrolyzed polysaccharide (Fig. 1, H) is very similar to that obtained with D-gala-L-mannoheptose (Fig. 1, G).

It was possible to adapt the well-known oxidation of aldo-sugars by bromine to a micro-scale procedure for distinguishing aldo- and keto-sugars:

.10 ml sugar (50 μg)
.35 ml 0.5M phosphate, pH 7.5
.05 ml 0.5M Br_2 in ethanol (or ethanol alone)

The above mixtures were incubated for 20 minutes at about 35°C. The pH dropped to about 6.9 during this time. To the bromine-treated sugar solution was added 0.5 ml of a similar mixture containing buffer, water and ethanol. To the control sugar solution (without bromine) was added 0.5 ml of a solution of water, buffer and bromine which had been preincubated 20 minutes to destroy the bromine. These 1 ml samples were then carried through the CyR10 procedure and the optical densities were measured at 410 mμ (for hexoses) or 510 mμ (for heptoses) about 2 hours after the addition of cysteine. It was necessary to compensate the bromine-treated and control sugar samples in the above manner with reagent blanks in order to equalize the effects of bromide ions on the CyR color reaction, since bromide may either augment or diminish the color depending on what sugar is being studied. The results obtained with several hexoses and heptoses are presented in Table I. It may be seen that the free heptose obtained from the phosphate-ester fraction of the hydrolyzed polysaccharide is oxidized to the extent of about 75% which is similar to results obtained with the other aldoses.

The bromide which forms during the incubation affects the color development in the CyR reaction in another manner. The yellow color given by hexoses is stabilized and does not shift to a blue on long standing such as occurs with most hexoses in the absence of

TABLE I. Effect of Bromine on Aldo- and Keto-sugars as Shown by the CyR Colorimetric Procedure. See *General methods* for details of procedure. Values are averages of duplicate determinations.

Sugar	Bromine	Wave-length (mμ)	Optical density
D-Glucose	—	410	.715
"	+	"	.058
D-Fructose	—	"	.996
"	+	"	.969
D-Gala-L-mannoheptose	—	510	.180
"	+	"	.016
L-Glucoheptulose	—	"	.272
"	+	"	.253
Heptose from polysaccharide	—	"	.176
"	+	"	.043

bromide ions. Similarly with the heptoses, a gradual shift in color to a purer pink is delayed in the presence of bromide. *Crystalline ethyl mercaptals* and their hexaacetates were prepared on a microscale similar to the procedure described by Wolfrom and Karabinos(6). It was possible to obtain a fair amount of crystalline derivatives with as little as 3 mg of heptose. In the case of the acetylated mercaptals, the extraction with chloroform was omitted and the derivatives were directly crystallized by the addition of water at the end of the acetylation reaction. The mercaptals were recrystallized from water and the acetylated mercaptals were recrystallized from ethanol by the addition of water. *Melting points* (uncorrected) were obtained with a Fisher-Johns melting point apparatus.

Experimental procedure. Polysaccharide material obtained from the somatic antigen of cells harvested from 16 carboys of a broth culture of *Sh. flexneri,* type 3, was refluxed for 4 hours at 100° C in N HCl. The solution was chilled and the pH was adjusted to about 8.5 by the addition of NaOH. Barium acetate was added and a small precipitate was removed. The supernatant solution was treated in the cold with 3 volumes of ethanol, the precipitate was washed with 80% ethanol and most was redissolved by the addition of a small amount of water and HCl to about pH 4. The insoluble residue was discarded and the supernatant fluid was readjusted to about pH 8.5 and the resulting precipitate was removed. The supernatant fluid was then treated with 3 volumes of ethanol and the precipitate was washed with 80% ethanol. This material was suspended in water and Ba was removed by the addition of H_2SO_4. The solution of phosphate esters was buffered with acetate at pH 5 and treated with seminal phosphatase. Approximately 50% of the phosphorus was released which represented about 65% of the heptose. Protein, inorganic phosphate and unsplit phosphate esters were removed by treatment with $ZnSO_4$ and Ba $(OH)_2$(7). The yellowish supernatant fluid was adjusted to about pH 5 by the addition of HCl and the solution was deionized by means of ion exchange resin columns. The deionized solution was adjusted to about pH 5 and was concentrated by vacuum distillation. The resulting solution was almost colorless and contained approximately 12 mg of heptose based on a comparison of the CyR color with that of D-gala-L-mannoheptose. The spectrum given by this material in the CyR10 reaction is shown in Fig. 1, H. Only one spot was obtained in one dimensional paper chromatograms in several solvents although the CyR4 spectrum showed the presence of a small amount of hexose which had previously been demonstrated in the phosphate ester fraction (1,3).

Failure to obtain a visible reaction of the heptose on paper chromatograms sprayed with orcinol(8) indicated the absence of a ketoheptose. That we were concerned with an aldoheptose was also shown by its oxidation by bromine (see *General methods*).

The concentrated HCl used in the prepara-

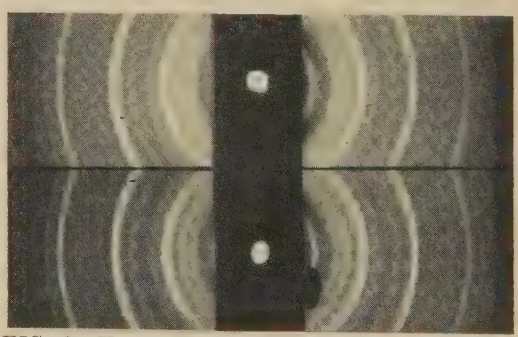

FIG. 2. X-ray diffraction patterns of crystalline hexaacetyl ethyl mercaptal of D-gala-L-mannoheptose (top) and the corresponding derivative of the heptose isolated from the polysaccharide of *Sh. flexneri* (bottom).

tion of mercaptals destroys ketoheptoses. We were able to prepare the crystalline ethyl mercaptal (plates) and hexaacetate of the ethyl mercaptal (needles) after drying the heptose solution obtained from the phosphate esters. Since the CyR spectra of the unknown heptose resembled that of D-gala-L-mannoheptose, the corresponding derivatives of the latter were also prepared. The ethyl mercaptals melted at about 200-203° C (uncorr.), but the mixed melting point was at 193-199°C. Similarly with the thrice crystallized hexaacetates of the ethyl mercaptals, the individually determined melting points were about 141-143° C, while the mixed value was lowered to about 133-138°C. The values for the separate derivatives agree fairly well with those reported for authentic samples of the D-gala-L-mannoheptose derivatives: ethyl mercaptal (204-205°C, corr.), acetylated ethyl mercaptal (145-146°C, corr.)(9).

Although D-altro-D-mannoheptose gives CyR spectra similar to those obtained with the heptose of the polysaccharide and with D-gala-L-mannoheptose (Fig. 1, F, G and H), the melting point of its ethyl mercaptal (151-153°C) is different(10).

X-ray powder diffraction patterns (Fig. 2.) show that the hexaacetyl ethyl mercaptal of the aldoheptose isolated from the polysaccharide is identical with that of gala-mannoheptose. However, such patterns do not differentiate between D and L configurations. Since we had very little material, it was not feasible to measure optical rotations, but the fact that the mixed melting points of both the mercaptals and the hexaacetyl mercaptals were lower than the individual melting points is a good indication that we are dealing with the enantiomorph, L-gala-D-mannoheptose.

Since ketoheptoses in the CyR reaction have a much higher absorption coefficient at 510 mμ than aldoheptoses (Fig. 1), it should be possible to detect isomerization of heptoses by measuring a change of optical density at this wavelength. Preliminary experiments with dialyzed sonic extracts of *Sh. flexneri* failed to reveal any isomerase activity with either the aldoheptose phosphate isolated from the bacterial polysaccharide or with sedoheptulose-7-phosphate kindly supplied by Dr. B. L. Horecker of the National Institutes of Health.

Discussion. This seems to be the first report of the occurrence of an aldoheptose phosphate in nature. Ketoheptose esters of phosphate have been reported previously(11-13), and free sedoheptulose (D-altroheptulose) and D-mannoheptulose were shown to be present in plants(15,16). It has recently been found that the polysaccharide of the somatic antigen of *Sh. sonnei,* phase II, contains a rather large amount of an aldoheptose apparently in non-phosphorylated form(14).

Summary. An aldoheptose phosphate has been isolated from the polysaccharide of the somatic antigen of *Sh. flexneri,* type 3. The heptose moiety is identical with gala-mannoheptose and appears to be the enantiomorph of D-gala-L-mannoheptose.

The authors wish to express their gratitude to Dr. Nelson K. Richtmyer of the National Institutes of Health for his interest in the problem of identification of the aldoheptose, to Mr. William White, also of the National Institutes of Health, for kindly preparing the X-ray diffraction patterns, and to Mr. William D. Hann for isolating the crude polysaccharide and other valuable assistance.

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Study with Fluorescent Antibody of Fate of Intradermally Injected Proteins in Rabbits.* (20232)

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The fate of intravenously injected antigenic substances has been followed with interest because of its bearing on the mechanism of destruction of foreign materials in relation to the stimulation of antibody formation, and the distribution of the lesions of experimental serum disease obtained with different antigens(1-4). The present report presents data concerning the sequence of events following intradermal injection of two antigenic proteins in normal and passively sensitized rabbits and in rabbits sensitized with homologous antigen plus Freund's adjuvants.

Methods. 2.5-3.0 kg rabbits were used throughout. Antiserum pools, containing 1.21 and 0.50 mg N/ml of antibody against Armour's crystalline ovalbumin (Ea) or Armour's bovine Fraction II (BGG), were obtained from animals injected with these antigens in combination with Freund's adjuvants. Some rabbits were passively sensitized the day before the test by intravenous injection of 4.0 mg of antibody N(5). Others were sensitized 3 weeks earlier by inoculating the two front foot pads with 0.05 ml of an emulsion containing 20 mg protein antigen and 3 mg heat-killed tubercle bacilli per ml of mineral oil (Bayol F). Test injections of 2 or 10 mg of 3 times recrystallized Ea(6) or BGG in 0.10 ml of physiological saline were made intradermally on the shaved outer aspect of the hind leg above the heel. Animals were sacrificed 1, 6, 24, and 48 hours after the test injection. The injected area of skin and the homolateral popliteal lymph node, and sometimes other tissues, were removed and quick frozen. 4-6 μ cold-box sections of these tissues were stained with fluorescein-labelled, homologous antibody, obtained from the serum pools described above, and examined under the fluorescence microscope. The technic of labelling antibody with fluorescein and the staining methods are described by Coons *et al.*(7-9). Controls demonstrating the specificity of the fluorescent staining included: absence of staining by heterologous labelled antibody, and inhibition of specific staining in sections treated first with unlabelled antibody. Sections stained by the Giemsa technic, were also examined.

Results. The normal rabbits showed minimal microscopic changes. Those sensitized passively or with the aid of adjuvants presented typical Arthus reactions, 10 to 25 mm in diameter, maximal at 6 or 24 hours, and remaining conspicuous at 48 hours in the adjuvant group. The microscopic picture included edema and polymorphonuclear exudation, marked at 1 hour in the deeper skin layers, involving all layers at 6 and 24 hours, and massive around vessels and hair follicles. Deep areas of hemorrhage were seen, particularly in the responses to Ea. At 48 hours, there was an increase in fixed cells and other mononuclears. The adjuvant sensitized rabbits showed more intense and prolonged reactions, including marked fixed cell activation in the papillary layer, some infiltration of lymphoid elements at 24 and 48 hours, and necrosis,

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† Fellow of the Canadian Arthritis and Rheumatism Society.

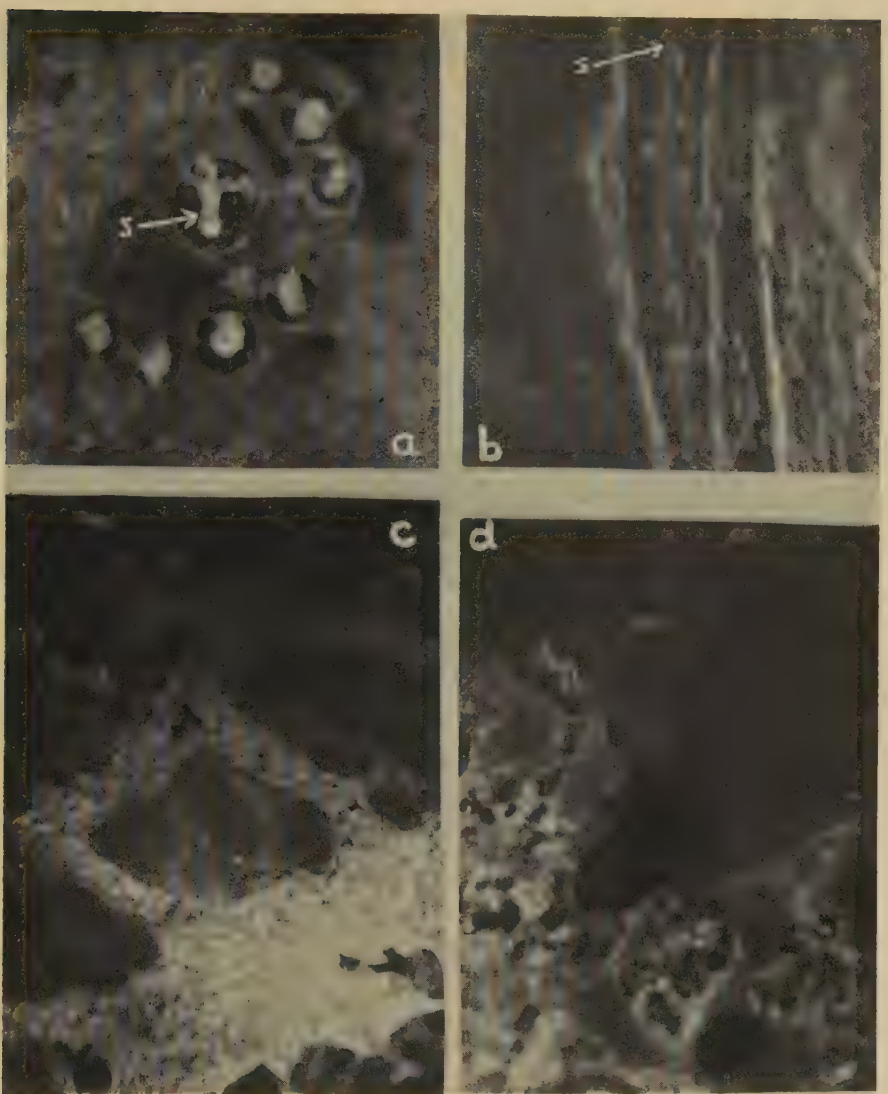

FIG. 1. One hr skin specimens, stained with fluorescent antibody. × 150. a. Ea, 2 mg, in normal rabbit. Antigen around and between hair follicle cells. Hair shafts (s) have own reddish fluorescence but no antigen. b. Gamma globulin, 2 mg, in normal rabbit. Hair follicles cut longitudinally. c. Gamma globulin, 2 mg, in passively sensitized rabbit. Antigen in deep connective tissue and around hair follicles. No antigen penetration into deep follicles themselves. d. Gamma globulin, 10 mg, in adjuvant-sensitized rabbit. Edematous connective tissue near deep follicles showing lakes of extracellular antigen.

especially of polymorphonuclears. The lymph node changes consisted of edema, hemorrhage, and polymorphonuclear infiltration involving the medullary and cortical sinuses and the nodules and parallel in degree to the changes in the skin. In the Freund sensitized rabbits, the reaction was prolonged and included extensive polymorphonuclear necrosis and loss of cortical tissue at 24 and 48 hours.

The amount of antigen present in the injected skin site and in the draining lymph node at various intervals after the test injection was evaluated according to both the intensity of staining with fluorescent antibody and the extent of antigen distribution. In the normal, Ea was almost entirely gone from the skin site at 24 hours and was transiently visible in the draining node at 6 hours. In the

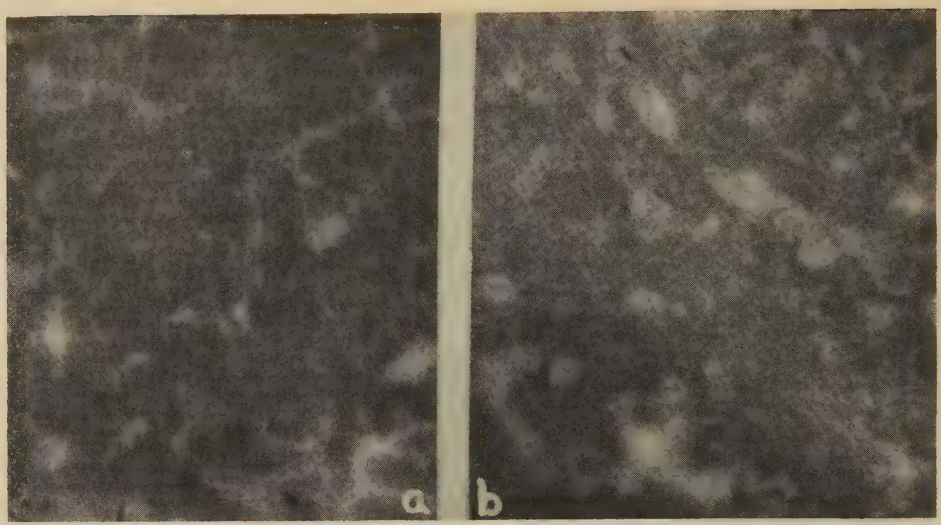

FIG. 2. Twenty-four hr skin specimens stained with fluorescent antibody, × 300. Both are Ea, 10 mg, in adjuvant-sensitized rabbits. a. Deep connective tissue, showing antigen between collagen bundles and contained in several histiocytic elements. b. Connective tissue immediately beneath epidermis, showing numerous antigen-containing macrophages.

passively sensitized animal, Ea disappeared from the skin as quickly as in the normal but was visible in the node both earlier (1 hour) and later (24 hours), as though the sensitizing antibody slowed its escape. In the adjuvant sensitized rabbits, this slowing effect was seen in both the skin site and the node, where more intense staining was also observed at all times. BGG, presumably because of its greater molecular size, disappeared slowly from the skin, where it was still found at 48 hours in normal as well as sensitized rabbits. It also disappeared slowly from the nodes and often gave more intense staining than the comparable Ea preparation. The fixation effect noted in Ea sensitized animals was also apparent in both skin and nodes of the rabbits sensitized to BGG.

At 1 and 6 hours, the antigen was spread widely in the deeper skin layers, while more superficially it was limited to the area immediately above the injection site. It was seen characteristically in large extracellular pools in the edematous deeper tissues and along the outside of hair follicles. It formed clearly defined borders along the collagenous connective tissue fibers as though adsorbed, and penetrated the hair follicles at an intermediate level to surround the hair shaft. The picture was identical for the 2 protein antigens studied, and is illustrated in Fig. 1. In the normal and passively sensitized rabbits, only a few cells of the histiocytic type, in the deep connective tissue, contained antigen in their cytoplasm at one and 6 hours. As antigen disappeared, however, from the follicles and the superficial skin layers first, small amounts could be demonstrated in macrophages (in cytoplasm and nuclei) near the skin surface. Antigen was not identified in the infiltrating polymorphonuclears; nor was there evidence of increased phagocytosis in the passively sensitized group. The last identifiable antigen to remain was usually seen in the deep connective tissue, and was still largely extracellular. In the adjuvant-sensitized animals, more phagocytosis was apparent in all parts of the tissue (Fig. 2), almost entirely by histiocytic elements containing antigen in nuclei as well as cytoplasm (some of these cells may have been fibroblasts). Antigen containing macrophages in the papillary layer were very conspicuous in this group, particularly at 6 and 24 hours. Here too the antigen was visible longest in the deep connective tissue, mostly in extracellular pools. None was seen at any time in the epidermis.

In the popliteal nodes of all groups of animals, antigen was found first in the peripheral and medullary sinuses and was extracellular.

Often a sinus bordering one lymphoid nodule contained a considerable amount of antigen while another quite close contained none. Later, much of the antigen was contained in reticular cells (in cytoplasm and nuclei) in the peripheral sinuses; and similar cells or small groups of cells were seen within the nodules. At the same time, considerable numbers of lymphoid cells at the periphery of the nodule showed antigen in their cytoplasm. No antigen was seen in the germinal centers. The sequence of events in passively immunized rabbits was the same as in normals. The whole process was speeded up and intensified in the adjuvant sensitized animals, most of the visible antigen being intracellular at 6 hours, while it remained largely in extracellular lakes in the normals. Such antigen as remained at 48 hours was entirely contained in histiocytic cells scattered throughout medulla and cortex.

Discussion. Protein injected into the rabbit skin behaves like protein injected by other routes in the mouse or rabbit. Coons and his collaborators(1) have shown that intravenously injected material rapidly appears in the connective tissue, where it is adsorbed on connective tissue fibers and taken up by reticulo-endothelial cells and possibly fibroblasts. It is not seen in epithelial tissue except in certain specialized organs, but is transiently present in lymphoid cells of spleen and lymph nodes. It disappears from the tissues at a rate apparently determined by its molecular size, and remains in reticulo-endothelial cells and connective tissue after disappearing from other sites. Thus, no characteristic distribution could be found to distinguish the intradermal from other routes of administration.

It was an unexpected finding of the present study that polymorphonuclear cells, which participated actively in the skin responses, did not contain antigen recognizable by the fluorescent antibody technic. Either these cells did not take up antigen or they destroyed it immediately after ingestion. In the normal rabbit ear chamber, dye-protein is taken up largely by macrophages, beginning at about 4 hours(10). In our skin sections, phagocytosis of antigen by macrophages was seen at one hour but was more definite at 6 hours, even in the adjuvant sensitized group.

It was surprising that the presence of homologous antibody in the passively sensitized rabbits did not bring about more obvious changes in the sequence of events. The great excess of antigen may have obscured any actual differences. In the passively sensitized group and in those animals sensitized with protein plus adjuvants, antigen was recognizable earlier and later at the local site and in the draining node. This finding suggests that diffusion of antigen into the general circulation was retarded, whether by the local edema or by combination with antibody. Otherwise it would appear inconsistent with the increased rate of antigen breakdown occurring in actively and passively immunized animals (11). The increased phagocytosis of antigen seen in the adjuvant group may correspond to the increased phagocytic capacity of histiocytic cells seen in the tuberculin type of sensitivity(12). The delayed, necrotic character of the skin reactions in this group of animals suggested that they may have depended in part on the tuberculin type of sensitivity.

Summary. 1. In the rabbit, intradermally injected crystalline egg albumin and bovine gamma globulin are taken up by histiocytic cells both locally and in the peripheral and medullary sinuses of the draining lymph node. Considerable quantities remain extracellular and presumably diffuse away to be dealt with elsewhere in the body. Polymorphonuclear cells, though present in considerable numbers, are not found to contain intact antigen. 2. In the draining node many lymphoid cells containing antigen are seen in the peripheral portions of the lymphoid nodules. 3. Egg albumin disappears much more quickly than bovine gamma globulin from the injected skin site and the draining node. 4. Passive sensitization and to a greater extent sensitization with Freund's adjuvants result in a slowing of both antigens' disappearance from skin and node. Adjuvant sensitized animals also show speeding up and intensification of phagocytosis of antigen in both localities.

The assistance of Dr. Albert Coons, Department of Bacteriology and Immunology, Harvard Medical School, is gratefully acknowledged.

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Clotting of Citrated Plasma by Bacteria which Destroy the Anticoagulant: Effect of Sodium Fluoroacetate. (20233)

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(Introduced by Reuben Kahn.)

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When certain bacteria are incubated in citrated plasma, they frequently remove the anticoagulant, citrate, and the blood clots (1,2). Clotting is a dependable end point of utilization of citrate and could offer a simple means of studying bacterial metabolism of citrate. The present study was conducted to clarify how the rate of bacterial utilization of citrate in plasma is related to bacterial growth and to clotting. In addition, sodium monofluoroacetate and potassium cyanide, substances known to affect metabolism of citrate in experimental animals, were tested for their action on the clotting of citrated plasma by bacteria.

Method. In all experiments, organisms were tested for clotting by inoculating 1.0 ml of citrated human plasma with 0.1 ml of a culture grown for 24 hours in tryptose phosphate broth. Inoculated plasmas were incubated at 37°C and examined daily to 8 days for clotting. Clotted plasmas were completely jelled. Unclotted plasmas remained entirely fluid. One ml of ACD solution (1.32 g sodium citrate, 0.48 g citric acid, and 1.47 g dextrose per 100 ml) was used for 4 ml of blood. Results were recorded only if heavy bacterial growth had occurred. Quantitative determinations of citrate were made on whole clotted plasmas, including fluid within the clot, by the method of Lardy(3). The *rate of clotting* of citrated plasma containing increasing concentrations of sodium monofluoroacetate or potassium cyanide was determined after inoculation with various strains of *Aerobacter aerogenes* or enterococci. The rate at which *bacteria destroyed citrate* was determined in mixtures containing 3 parts of citrated plasma

TABLE I. Clotting by Bacterial Consumption of Citrate. Results obtained by growth of 116 strains in citrated blood.

Bacteria	Strains tested, No.	Strains which clotted citrated human blood, No.
A. aerogenes	18	16
A. cloacae	2	2
K. pneumoniae	8	6
Salmonella species	5	4
Diphtheroid	2	2
Ps. aeruginosa	15	7
B. alcaligenes	1	1
Paracolon species	4	2
E. freundii	8	3
E. coli	20	12
Shigella species	2	1
Staphylococcus coagulase—	15	2
Enterococcus	13	4
Str. viridans	3	0
Totals	116	62

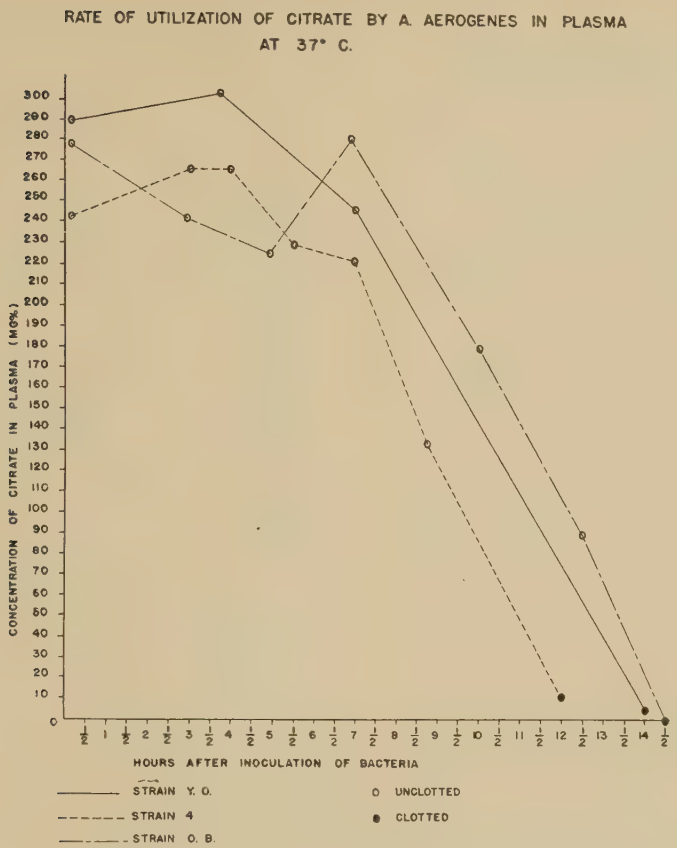

FIG. 1. Rate of utilization of citrate in plasma by *A. aerogenes* and relationship to clotting.

and 1 part of a 24 hour broth culture of *A. aerogenes*. These inoculated plasmas were divided into aliquots which were incubated simultaneously at 37°C. Individual aliquots were removed at intervals from the incubator and rapidly frozen. The aliquots were later thawed and examined for citrate content. Bacterial destruction of citrate was also determined in aliquots containing 10 mg of fluoroacetate per ml of clear plasma which had been examined turbidimetrically in the Coleman spectrophotometer for bacterial growth. *Anaerobic* studies were conducted in jars whose air had been evacuated and replaced by helium.

Results. The occurrence of clotting in plasma inoculated with any of 116 strains of bacteria is shown in Table I. Clotting was characterized by sudden jelling of the entire sample of plasma. The clot did not retract for 24 hours. Results with coagulase-positive staphylococci are omitted because the coagulase reaction masked clotting resulting from removal of citrate. The concentration of citrate after plasmas were clotted by strains of enterococci and *A. aerogenes* was frequently zero and usually less than 30 mg %. These bacteria did not clot heparinized plasma, oxalated plasma, serum or fibrinogen solution.

The rate of utilization of citrate with 3 strains of *A. aerogenes* is shown in Fig. 1. Citrate concentration falls abruptly after a stationary period when relatively little change is observed. Bacterial growth curves in Fig. 2 illustrate that the sharp fall in citrate concentration takes place after the bacteria have been in the stationary growth phase for about 4 hours. A secondary increase in bacterial density (A-B in Fig. 2) appears during the period of rapid destruction of citrate. This secondary rise in bacterial density, presumably due to bacterial growth, was sharply reduced

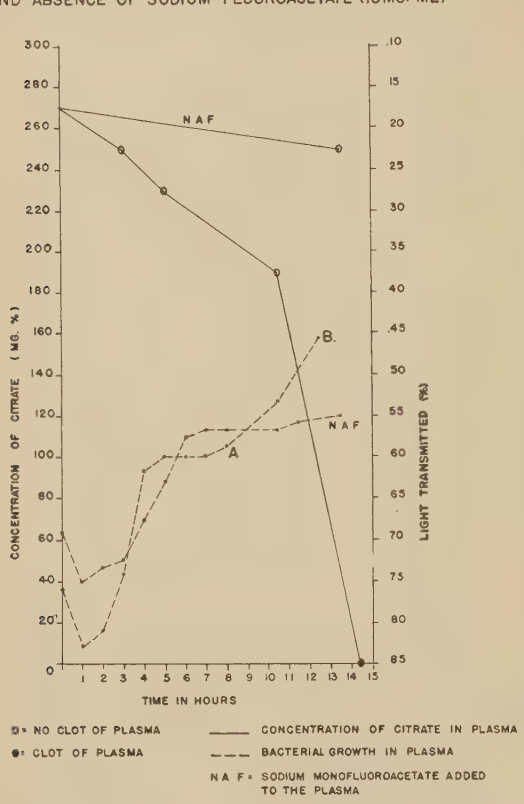

FIG. 2. Comparison of growth and citrate utilization in citrated plasma by *A. aerogenes* in presence and absence of sodium fluoroacetate (10 mg/ml).

by fluoroacetate at the same time that clotting and citrate utilization were prevented.

Fluoroacetate inhibited the clotting produced by many strains of both *A. aerogenes* and enterococci. As shown in Fig. 3, delay of bacterial clotting bore almost a linear relation to the concentration of fluoroacetate. Fluoroacetate itself was no anticoagulant and did not change pH. Complete anaerobiosis did not delay clotting produced by 13 strains of *A. aerogenes,* but repeatedly abolished or reduced the inhibitory action of fluoroacetate (5.0 g per ml) on bacterial clotting. Whenever clotting was prevented by fluoroacetate, the utilization of citrate was found to be inhibited.

The presence of 0.1 M KCN inhibited clotting of citrated plasma by 8 strains of *A. aerogenes,* but did not inhibit clotting by 11 strains of enterococci. Surprisingly, KCN actually hastened clotting by 6 strains of enterococci, although KCN has no effect of its own on clotting.

Discussion. The evidence presented here demonstrates that clotting of citrated plasma may be used as a dependable and simple end point of citrate utilization in studying bacterial metabolism. Under controlled conditions, a given strain can be depended upon to clot citrated plasma after uniform periods of incubation.

Data obtained in this study by observing bacterial clotting, and supported by measurement of citrate, indicate that sodium monofluoroacetate inhibits citrate utilization by *A. aerogenes* and enterococci. Fluoroacetate was examined for its effect on clotting of citrated plasma by bacteria because it has been found to prevent citrate utilization in animals. It has been postulated that fluoroacetate prevents oxidation of citrate in the tricarboxylic acid cycle(4,5). It is possible therefore that in preventing utilization of citrate by *A. aerogenes,* fluoroacetate acts in an oxidative cycle resembling the citric acid cycle. The loss by fluoroacetate of its inhibitory effect on clotting under anaerobic conditions further indicates that fluoroacetate activity was related to oxidative bacterial metabolism.

In the case of enterococci, however, it would be unlikely that an oxidative cycle of the tricarboxylic type is involved in utilization of citrate. Enterococci possess no iron-enzymes, and it was not surprising to discover that KCN did not prevent or delay clotting by these bacteria. The marked inhibitory effect of fluoroacetate on clotting by enterococci suggests that the antagonistic action of fluoroacetate to citrate utilization is not limited to oxidative systems resembling the conventional citric acid cycle.

Summary and conclusions. The relationship of bacterial growth to utilization of citrate and clotting of plasma has been determined. Clotting of citrated plasma may be used as a dependable and simple end point of citrate utilization by certain strains of bacteria. Fluoroacetate was found to prevent clotting by interfering with utilization of citrate by *A. aerogenes* and the enterococci.

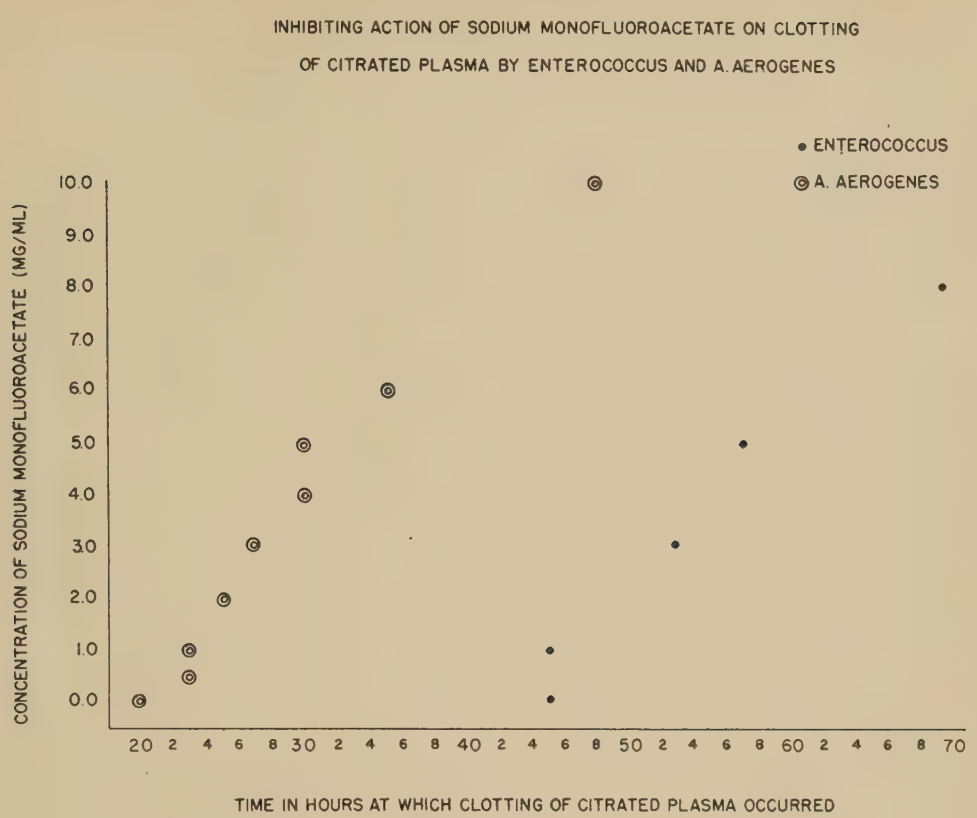

FIG. 3. Inhibiting action of sodium monofluoroacetate on clotting of citrated plasma by enterococcus and *A. aerogenes*.

Cyanide had no effect on clotting by enterococci.

The sodium fluoroacetate was generously provided by Dr. Maynard Chenoweth.

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Insulin Sensitivity in the Hereditary Hypopituitary Dwarf Mouse.* (20234)

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Snell(1) described in a strain of mice an hereditary dwarfism which behaved as a true Mendelian recessive. Since then many investigations have been carried out on the dwarf which have shown interesting morphological and metabolic anomalies(2,3). Russell (4) reported that hypophysectomized or adrenalectomized animals exhibit a greatly increased sensitivity to the hypoglycemic effect of insulin as compared to normal intact animals.

Since the hereditary dwarf is deficient in anterior-pituitary and adrenocortical secretions, it was of interest to test insulin effects on this naturally occurring form.

Materials and methods. The animals used in this study were hereditary hypopituitary dwarf mice of the Bar Harbor strain (Subline, Syracuse); for controls and comparative purposes, normal mice of the same strain were utilized. All dwarfs used were 9 to 12 months old, ranging in weight between 9 and 14 g. Normal mice between 35 and 42 days old were used as controls because their weights at this time were similar to those of the dwarfs; moreover, at this age the normals are developmentally similar to the dwarfs at 9 to 12 months. The animals were fed Purina Laboratory Chow and water *ad lib.* The temperature of the animal room was carefully maintained at 75°F. Insulin (Illetin-Lilly) was administered subcutaneously.

Results. Insulin sensitivity. The dwarfs are remarkably more sensitive to insulin than normal mice, for it takes only 3% of the dose of insulin given to normal mice (2 units per kilo) to cause a similar convulsive effect. For instance, a single dose of 0.06 unit of insulin injected after the 24th hour of fasting is sufficient to cause in 1 hour a marked decrease of 45% in the blood sugar level. The same dosage of insulin causes a slight but significant drop in the blood sugar of normal mice (Table I). When dwarfs are fasted for 18 hours, allowed food for 6 hours, and then injected immediately with 0.06 unit of insulin per kilo, the blood sugar level drops 23% in an hour, or about half as much as was observed above following a 24-hour fast. In contrast, normal mice under similar experimental conditions of dosage and fasting exhibit a decrease of 9% (Table I). Further evidence of this marked insulin sensitivity of the dwarf is provided by the observations that higher insulin dosages of 0.125, 0.250, and 0.500 unit of insulin per kilo cause hypoglycemic coma and death in the dwarfs but produce no observable symptoms such as hypoglycemic cramps in the normal controls. When after 24 hours of fasting, adrenalectomized mice of normal size are given 0.5 unit of insulin per kilo, cramps and hypoglycemia result in 1 hour; but the animals do not succumb. If the dosage is raised to 2 units per kilo, the adrenalectomized animal enters a hypoglycemic coma which is followed by death.

Anti-insulin tests. In Table II the effects of various hormones acting as anti-insulin agents are indicated. Adrenocorticotrophin, cortisone, and adrenocortical extract all exhibit a marked capacity to prevent convulsions. Cortisone exerts the most marked anti-insulin response in both normal and dwarf mice. In normal intact animals cortisone, ACTH, and adrenocortical extract counteract the effects of insulin to such an extent that 5, 4, and 3 times the dose of insulin respectively are necessary to produce approximately the same percentage of convulsions that occurs in nonprotected normal controls receiving 2 units of insulin per kilo. If normal mice are adrenal-

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TABLE I. Effect of 0.06 Unit of Insulin per Kilo on Blood Sugars in Fasted Dwarf (dd) and Normal (N) Mice.

	Treatment	No. animals	Mean blood sugar, mg % ± S.E.,* at hr fasted						
			24†	25	26	28	32	40	45
dd	Insulin (.06 ml)	10	96±2.1	53±1.8	72±2.2	75±3.0	89±0.9	94±1.0	90±1.3
N	Idem	11	128±3.2	114±2.0	117±1.0	127±1.7	124±1.3	119±1.2	121±2.8
	Data in lower half of table similar to above except that animals were fasted 18 hr, fed between 18th and 24th hr, and fasted again after 24th hr.								
dd	Insulin (.06 ml)	6	146±4.1	113±1.1		90±2.4			82±1.8
N	Idem	6	150±3.8	137±2.0	142±1.9	144±3.0	130±0.9	121±2.6	112±1.2

* S.E. = stand. error. † Insulin inj. subcut.

TABLE II. Anti-Insulin Tests in Normal Intact and Adrenalectomized Mice and Intact Dwarfs Fasted 24 Hr.

Treatment	Amt inj.	Insulin dose/kilo mouse	Total No. animals	Convulsions, %
Intact normal mice				
Controls	—	2	39	90
ACTH	.5 mg	2	14	14
		8	10	80
Cortisone	.2 "	2	18	0
		10	10	70
ACE	.7 ml	2	22	14
		6	10	90
DCA	.2 mg	2	19	84
	2.0 "	2	10	100
Testosterone propionate	2.5 "	2	21	100
Adrenalectomized normal mice*				
Controls	—	.5	9	100
ACTH	.5 mg	.5	8	88
		2.0	5	70
Cortisone	.2 "	.5	10	0
		2.0	5	80
ACE	.7 ml	.5	10	10
		2.0	5	100
Intact dwarf mice				
Controls	—	.06	12	100
ACTH	.5 mg	.06	6	17
		.25	5	80
Cortisone	.2 "	.06	10	10
		.30	5	60
ACE	.7 ml	.06	12	17
		.25	5	80
DCA	.2 mg	.06	12	100
	2.0 "	.06	12	100
Testosterone propionate	2.5 "	.06	12	100

* Adrenalectomized animals maintained on daily subcut. inj. of 0.1 mg of DCA in 0.1 ml of peanut oil and 0.9% sodium chloride in drinking water.

ectomized and primed with cortisone and ACE, 2 units per kilo instead of 0.5 unit per kilo are required to cause convulsions. Dwarf mice receiving ACTH, cortisone, and ACE convulse with 4, 5, and 4 times the dose of insulin respectively in contrast to nontreated control dwarfs receiving 0.06 unit of insulin per kilo.

The results of these experiments (Table II) also serve to emphasize the ineffectiveness of DCA as an anti-insulin agent in both normal and dwarf mice, since dosages from .2 to 2

mg proved incapable of preventing convulsions. This observation is of interest since it has been generally considered by Wang and Verzar(5) that DCA is capable of affecting carbohydrate metabolism. With a tenfold increase in the dosage, it appears that sufficient DCA absorption should occur to reveal any possible anti-insulin effect. Nevertheless, none was obtained.

In all experiments reported here, testosterone propionate exhibited no anti-insulin effect (Table II).

Discussion. Although this study has shown that the hypopituitary dwarf mouse is extremely sensitive to insulin as compared with controls, the mechanism to explain the increased sensitivity of the dwarf is not definitely understood. On theoretical grounds, any or all of the following factors may be responsible for this phenomenon: a) A decreased rate of inactivation of insulin by the blood and tissues. b) Inadequate counterregulatory responses (*i.e.*, glycogenolysis and gluconeogenesis) to hypoglycemia by the liver. c) Hypophyseal and adrenocortical deficiencies, perhaps rendering the dwarf incapable (especially under the stress of fasting) of producing an anti-insulin factor or factors such as ACTH and adrenocortical hormones oxygenated at C-11 and 17.

Identification of insulin-antagonistic factors is of interest. The present study shows that ACTH, cortisone, and ACE are antagonistic to the blood-sugar lowering action of insulin, *i.e.*, they increase the tolerance of the dwarf to a given dose of insulin. Preliminary experiments (unpublished data) have shown that growth hormone is also anti-insulin. Insulin sensitivity and anti-insulin action under these conditions perhaps are associated with a pituitary deficiency *per se* as well as with the deficiencies of the pituitary-adrenal axis. The above findings are consistent with what is known in other experimental and clinical situations(6-8).

Summary. Under the experimental conditions employed in these studies, findings may be summarized as follows: a) The insulin-hypersensitive hereditary hypopituitary dwarf mouse can tolerate only 3% of the dose of insulin that produces comparable symptoms in normal mice (2 units of insulin/kg of mouse). Higher dosages cause severe hypoglycemia followed by death. b) ACTH, adrenocortical extract (ACE), and cortisone act as anti-insulin agents as shown by their ability to increase blood-glucose levels and to reduce hypoglycemic convulsions. Desoxycorticosterone acetate and testosterone propionate exhibit no anti-insulin action. c) Factors that may be responsible for the hypersensitivity to insulin are discussed.

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Enhancement of Adrenocorticotrophic Activity. (20235)

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The effects of adrenocorticotrophin, administered parenterally, in water, are submaximal. The failure to achieve full utilization of the hormone, so given, has been ascribed to destruction of the material at the injection site (1,2). Raben *et al.*(3) found that oxycellulose purified ACTH had twice the effect of the crude material when injected in similar vehicles and have suggested that destructive enzymes, originally present, have been removed during the process of purification of the hormone preparation. Wolfson *et al.*(4) have shown that ACTH administered in heavy gelatin resulted in a twofold increase in clinical effectiveness as compared to that administered in water solution. Solem(5) and Fletcher(6) have prolonged the duration of ACTH action by means of protamine zinc complexes and beeswax in oil respectively. The effectiveness of these methods has been attributed to 1) a slow release from an adsorbed state or a chemical complex and 2) a sequestering effect of the hormone from the tissues with concurrent slow release(4). Recently, Hamburger(7) has shown that polyphloretin phosphate is capable of prolonging the effects of ACTH when administered intramuscularly to rats, and Hogberg *et al.*(8) reported clinical data demonstrating the increased effectiveness of such preparations.

Polyphloretin phosphate has been shown to have antihyaluronidase action(7). This suggested the evaluation of other substances possessing similar properties as a method of prolonging the action of parenterally administered ACTH. In view of the finding by Hamburger(7) that polyphloretin phosphate protected ACTH from *in vitro* inactivation by serum, the substances described below were also examined for antiproteolytic activity.

Material and methods. Sprague-Dawley male rates, 110-115 g body weight, were hypophysectomized by the parapharyngeal approach 24 hours before the experiments. Injections were given subcutaneously in 0.5 ml volume. Purified corticotropin (Princeton), 25 U.S.P. units/mg, was incorporated into the solutions of substances to be tested so that the final solution contained 2 U.S.P. units/ml. Each experimental animal therefore received 1 U.S.P. unit of adrenocorticotrophin. Control animals received injections of equal volumes of the test solutions without ACTH. Adrenal ascorbic acid concentration was determined on the left gland, 3 hours, and on the right gland, 6 hours after injection. The ascorbic acid concentrations were determined by the method of Mindlin and Butler(9). The following substances were tested for their effect on ACTH: 0.2% suramin; 5% phosphorylated hesperidin; 2.5% phosphorylated hesperidin; 5% hesperidin methyl chalcone; 15% gelatin; 15% gelatin plus 20 T.U. Hyaluronidase and 15% gelatin plus 4% phosphorylated hesperidin. All solutions were adjusted to pH 4. Hyaluronidase, when used, was added immediately before injection. For the *in vitro* experiments on anti-proteolytic activity; suramin, phosphorylated hesperidin, and hesperidin methyl chalcone, were tested for possible inhibition of tryptic digestion of casein. In the *first experiment,* trypsin (Princeton), phosphorylated hesperidin solutions of mole ratios 1:2 and 1:4 (0.0006% and 0.001%), were prepared at pH 7.4. Neurath(10) has described a trypsin: diisopropylfluorophosphate complex at mole ratio 1:3 with almost complete loss of enzyme activity. The activities were measured immediately and after incubation at 37°C for 150 minutes. A *second experiment,* to approximate the concentrations of the test compounds used in the ACTH assay, involved the preparation of trypsin solutions in 2.5% and 5% phosphorylated hesperidin and hesperidin methyl chalcone, and 0.2% suramin. Tryptic activity was measured immediately and after 21 hours at 5°C. The method of Anson(11) was used throughout, with appropriate blanks for the materials tested.

TABLE I. Effect of Various Materials Upon ACTH Induced Adrenal Ascorbic Acid Depletion at 3 and 6 Hr Post Injection.

Vehicle %		No. of animals	Dose of ACTH U.S.P. unit	Adrenal ascorbic acid mg/100 g adrenal gland 3 hr	6 hr
			A.		
	H_2O	5	1	279 ± 11	314 ± 6
5	p.h.*	5	0	398 ± 14	383 ± 17
5	p.h.	10	1	180 ± 9	231 ± 13
2.5	p.h.	5	1	175 ± 7	245 ± 14
5	h.	5	0	404 ± 16	353 ± 21
5	h.	5	1	304 ± 16	322 ± 13
0.2	s.	5	1	231 ± 9	315 ± 14
15	g.	10	1	178 ± 10	240 ± 15
15	g. + 10 T.U. hy	5	1	212 ± 17	271 ± 10
			B.		
15	g.	5	1	165 ± 8	230 ± 9
15 g + 4%	p.h.	5	1	261 ± 16	152 ± 8

* p.h. = phosphorylated hesperidin; h = hesperidin methyl chalcone; s = suranim; g = gelatin; hy = hyaluronidase. All values are means ± standard error.

Results and discussion. The data presented in Table IA indicate that a subcutaneous dose of ACTH can be more effectively utilized in the rat if administered in a medium which delays absorption of the hormone. Phosphorylated hesperidin and heavy gelatin seem to be the most effective single agents, of those tested, in producing such a delay. However, a combination of these materials results in an augmentation of effectiveness, with the maximum response delayed from the third hour to the sixth hour post injection. Further studies extending the period of examination to 12 and 24 hours are now in progress. These substances conceivably act by different mechanisms to produce the prolongation of activity.

The phosphorylated hesperidin probably acts by inhibiting tissue hyaluronidase. The correlation of the efficacy of phosphorylated hesperidin in prolonging the action of ACTH, with the anti-hyaluronidase properties of the compound(12), indicates that the tissue spreading factor is involved. The methyl chalcone of hesperidin, which has no significant anti-hyaluronidase properties(12) has no enhancing effect on ACTH, despite its inhibition of proteolytic activity, *in vitro,* equivalent at the concentration used, to that of phosphorylated hesperidin.

In vitro studies with suramin and the hesperidins showed that both the methyl chalcone and phosphorylated hesperidin, as well as suramin are capable of inhibiting the proteolytic activity of trypsin (Table II). Inasmuch as both of these hesperidins exhibit similar degrees of anti-proteolytic effect, whereas only the phosphorylated hesperidin shows enhancing properties when combined with ACTH, it is concluded that the mechanism involved in the latter phenomenon is probably an action on the spreading factor. Unfortunately, suramin could not be used in larger doses *in vivo* due to its toxicity, and therefore its effect in prolonging the action of ACTH could not be measured at a concentration similar to that

TABLE II. Inhibition of Tryptic Digestion of Casein.

Material	% conc.	Hr of contact of trypsin with test substance before digestion with casein	°C	% inhibition†
P.h.*	.0006	.0		0
"	.0006	2.5	37	29
"	.0012	.0		0
"	.0012	2.5	37	71
	5.0	.0		50
"	2.5	.0		49
"	2.5	21	5	68
H	5.0	.0		58
"	2.5	.0		23
"	2.5	21	5	25
S	.2	.0		0
"	.2	21	37	83

* P.h. = Phosphorylated hesperidin; H = hesperidin methyl chalcone; S = Suramin.

† As compared to activity of trypsin solutions of equal concentration, under same conditions, in acetate buffer without test substances.

used for phosphorylated hesperidin. In the data obtained with the corticotrophin in gelatin plus phosphorylated hesperidin (Table IB) a summation effect is evident. There is the possibility that here the anti-proteolytic effect of the phosphorylated hesperidin (Table II) may also be a factor in that the gelatin is not acted upon by tissue proteinases and thus the gelatin retains its depot effect for a longer period of time. Thus the overall action of the gelatin-phosphorylated hesperidin combination may be the result of an increased depot effect of the gelatin due to the anti-proteolytic action of the phosphorylated hesperidin as well as the anti-hyaluronidase effect of this compound. The findings discussed above point to the use of a depot agent—anti-hyaluronidase vehicle for extending the duration of effect of other parenterally administered substances.

Preliminary clinical studies confirm the animal data in that the effectiveness of ACTH administered in phosphorylated hesperidin is as effective if not superior to gelatin preparations.

Summary. 1. Subcutaneous administration of purified corticotrophin in gelatin; in phosphorylated hesperidin; and in gelatin plus phosphorylated hesperidin results in an enhanced effect of the hormone upon the adrenal cortex as measured by adrenal ascorbic acid. The combination of gelatin plus phosphorylated hesperidin has been shown to be most effective in extending the duration of effect of the corticotrophin. 2. The mechanism of action is attributed to the antihyaluronidase as well as the anti-proteolytic properties of the phosphorylated hesperidin.

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Age Susceptibility Pattern of the Rat to Epidemic Keratoconjunctivitis Virus. (20236)

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Recent investigations(1,2) indicate that the viruses of epidemic keratoconjunctivitis (EK) and St. Louis encephalitis (SLE) are closely related. The two viruses would appear to be so closely related that it is not possible to differentiate them by serological methods. By studying the host range of the two viruses it was noted, however, that the viruses are not identical. It was observed(3) that while both viruses induce a fatal encephalitis in mice only the EK virus was capable of initiating a fatal encephalitis in guinea pigs and rabbits. It was also observed that EK virus, like SLE, does not produce any clinical evidence of infection in young adult rats following intracerebral inoculation of the virus(3). Since it has already been demonstrated that 7- or 8-day-old rats are highly susceptible to SLE virus,

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TABLE I. Susceptibility of Rats of Different Ages to Epidemic Keratoconjunctivitis Virus Injected Intracerebrally.

Age	No. tested	Wt		Results		
		Avg, g	Range, g	Mortality Total	Mortality %	Day of death*
7 days	6	13.5	11– 16	6	100	5, 7, 7, 7, 8, 8
7	5	12.7	12– 14	5	100	6, 6, 6, 6, 7
12	2	14.5	14– 15	0	0	S, S
12	9	20.2	16– 22	7	78	7, 7, 7, 7, 8, 8, 9, S, S
3 to 4 wk	16	43.0	40– 50	0	0	S (16)†
7 to 8 wk	16	141.3	126–162	0	0	S (16)†

* Numeral indicates day of death; S = survival.
† S (16) = 16 survived.

whereas rats 21 days of age or older are resistant, as far as clinical infection is concerned (4,5), studies were conducted to determine whether rats manifest a similar age susceptibility pattern toward EK virus and thus to obtain further information regarding the relationship between the 2 viruses.

Materials and methods. Virus and animals. The strain of EK virus used in the studies was obtained from Dr. Isaac Ruchman. A single virus suspension was used and was prepared from virus-infected brains of 3- to 4-week-old Swiss mice. The infected brains were homogenized in a Waring blendor together with sufficient inactivated, undiluted rabbit serum to make a 10% suspension of the infected brains. The homogenate was centrifuged at about 2000 RPM for 5 minutes to sediment gross particles. The supernatant fluid was removed and immediately distributed in 2 ml amounts in screw-capped vials. The virus suspension in the vials was "quick frozen" by rolling the vials in a mixture of dry-ice and alcohol. The frozen virus was then stored at about –70°C in a dry-ice cabinet. When needed it was thawed by placing a vial of the frozen virus in a 37°C water bath. The mouse intracerebral LD/50 titer(6) of the virus suspension, after storage at about –70°C for 4 days, was 1 x $10^{-8.3}$. The rats used in the studies were obtained from Sprague-Dawley, Madison, Wisc. All rats which were younger than 21 days of age when tested were reared in this laboratory and an accurate record of their ages was available. Older rats were shipped when 21 days of age. All rats inoculated when they were younger than 21 days of age were allowed to remain with the mother until they were at least 21 days of age. The rats were weighed on the day they were inoculated and the average weight and weight range of each group determined. All rats were observed twice daily for 21 days after inoculation of the virus.

Experimental. Behavior of rats of different ages following intracerebral inoculation of the virus. Groups of rats varying from 7 to 52 days of age were injected intracerebrally with a 10% suspension of virus. All animals received 0.03 ml of virus except the 7- to 8-week-old rats which received 0.1 ml, 0.05 ml being injected into each cerebral hemisphere. The results appear in Table I. All of the 7-day-old animals developed a fatal encephalitis. In these young animals the first indications of infection, abdominal distention together with weakness of the posterior extremities and a tendency to fall to one side when walking, were seen between the 4th and 5th days following inoculation. They also showed humped (hyperflexed) backs and became hyperexcitable, frequently biting the handler. Terminal signs were emaciation, convulsions, prostration, and in one instance paralysis of the posterior extremities. The 2 litters tested when 12 days old showed different degrees of susceptibility. A fatal encephalitis developed in 7 rats of a litter of 9, whereas both rats of a litter of 2 survived. It should be noted that the smallest animal in the litter of 9 weighed more than either animal in the smaller litter. The four 12-day-old rats which survived apparently did not completely escape infection. Although never showing characteristic signs of infection with EK virus, their fur was ruffled and they failed to grow at a normal rate during the 4- to 5-day period during which the other 12-day-old rats either showed characteristic signs or died. The course of the dis-

TABLE II. Susceptibility of Young Rats to Epidemic Keratoconjunctivitis Virus Instilled into the Nose.

Age, days	No. in each litter tested	Wt		Results	
		Avg, g	Range, g	% mortality	Day of death
7	6	9.2	8 –11	100	5, 6, 7, 8, 8, 8
7	2	19	18.5–19.5	100	7, 8
7	1	16	—	100	9
12	12	19.1	18 –21	100	8 on 8th day, 4 on 9th day

ease in the 12-day-old rats, which developed a fatal encephalitis, was essentially the same as that noted for the 7-day-old animals. All of the 3- to 4-week-old rats as well as all of the 7- to 8-week-old animals appeared to be resistant in that none of these animals developed a clinically apparent infection.

Behavior of 7- and 12-day-old rats following nasal instillation of the virus. Since 7-day-old rats were uniformly susceptible to EK virus injected directly into the brain and because some 12-day-old rats were found to be susceptible when the virus was so injected, an experiment was done to determine the behavior of young rats following intranasal inoculation of the virus. The results appear in Table II and show that both age groups were uniformly susceptible. The data presented in Tables I and II might suggest that 12-day-old rats are more susceptible to intranasally inoculated virus than to virus injected directly into the brain. Another possible explanation may be that at or about 12 days the susceptibility pattern changes so that in any large group of rats of this age some would be found to be resistant and others susceptible regardless of whether the virus were inoculated intranasally or intracerebrally. A similar observation has been reported for the behavior of Japanese B encephalitis virus in 12-day-old rats(7). The signs observed in the 7- and 12-day-old rats, which had the virus instilled into the nose, were the same as those already noted for 7-day-old rats which had been inoculated intracerebrally.

Discussion. It is apparent from the data presented that very young rats are susceptible to clinical infection with epidemic keratoconjunctivitis virus following either intracerebral or intranasal inoculation of the virus. As rats grow and develop, however, they, become increasingly resistant so that when they are 3 to 4 weeks of age they are no longer susceptible even though the virus is injected directly into the brain. Duffy and Sabin have reported a similar age-susceptibility pattern for St. Louis encephalitis virus(4,5). Seven- and 8-day-old rats were found to be susceptible to St. Louis encepalitis virus when the virus was inoculated by either the intracerebral or intranasal route. On the other hand 21-day-old rats were found to be refractory to the virus in that they did not develop a clinically apparent infection when St. Louis encephalitis virus was injected directly into the brain.

It is evident, therefore, that the age-susceptibility pattern of the albino rat to both epidemic keratoconjunctivitis virus and St. Louis encephalitis virus is similar because 7-day-old rats are uniformly susceptible to both viruses and a high degree of susceptibility is seen in 12-day-old rats. However, rats 3 to 4 weeks of age appear to be uniformly resistant to clinical infection with either virus in that rats of this age fail to develop a clinically apparent infection even though the viruses are injected by the intracerebral route.

Summary. 1. A uniformly fatal encephalitis develops in 7-day-old rats following either intracerebral injection or intranasal instillation of epidemic keratoconjunctivitis virus. 2. Most of the 12-day-old rats tested were found to be susceptible to infection with epidemic keratoconjunctivitis virus but were not uniformly so since some animals in this age group survived. 3. Rats 3 to 4 weeks of age as well as rats 7 to 8 weeks of age apparently are refractory to epidemic keratoconjunctivitis virus since they did not develop a clinically apparent infection following intracerebral injection of the virus.

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Production of Generalized Shwartzman Reaction with Group A Streptococcal Factors.* (20237)

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Recent studies by Thomas, Denny and Floyd(1-3) suggest that the mechanisms which bring about the generalized Shwartzman reaction may play a role in the pathogenesis of rheumatic fever and other disorders. Their studies involve the preparation of rabbits for the generalized Shwartzman reaction by infection with group A streptococci and provocation of the reaction by the injection of toxins from Gram-negative organisms. They observed that heat-killed group A streptococci and culture filtrates did not possess the property of preparing rabbits for this reaction and it was necessary to utilize products of Gram-negative organisms for the provoking injection. This paper is concerned with observations which indicate that when group A streptococci multiply in an *in vivo* environment a soluble material is produced which possesses the property of preparing rabbits for the generalized Shwartzman reaction. This reaction may be provoked in rabbits, prepared with this factor, not only by toxins from Gram-negative bacteria, but, in addition, by reduced filtrates of group A streptococcal cultures containing active streptolysin O.

Materials and methods. Streptococcal skin lesion extract. The preparation and extraction of this lesion extract was described by Watson and Cromartie(4). This technic consists of intracutaneous injection of 30 ml of an 18-hour Todd-Hewitt broth culture of type 28 group A streptococci in about 30 sites over the shaved abdomen and thorax of 2 kg New Zealand white rabbits. The moribund animals were killed 24 hours after injection of the streptococci, the lesion ground in a meat grinder and extracted overnight at 2°C by stirring with 1 to 2 ml of saline per g of tissue. This was filtered through gauze and centrifuged in the Spinco 30 head at 25,000 rpm for ½ hour. This supernatant was filtered through a Selas bacterial filter and kept frozen in sealed ampules at –70°C. Four lots of this material were used in the work reported in this paper. They are referred to as streptococcal lesion extract 108, 109, 112, or 116. The total nitrogen of extracts 108 and 109 was 1.7 mg N per ml. Extract 116 contained 3.24 mg N per ml. As a preparing dose 0.5 ml of extract 108 or 109 was used. A preparing dose of extract 116 was 0.25 ml. Lesion extract 112 was prepared as above and was then concentrated 10 fold by lyophilization and dialyzed against physiological saline for 24 hours.

Bacterial toxins. Typhoid toxin was prepared from N-28-1 strain of *Salmonella typhosa*. The organism was cultured 9 days in nutrient broth under semi-anerobic conditions. It was then filtered through a Selas bacterial filter and stored in the refrigerator under aseptic conditions Six lots of the toxin were produced. The potency of each lot was

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determined by the injection of 6 rabbits with doses varying from 1.0 ml to 4.0 ml. The toxicities of all preparations were quite constant. Three to 4 ml were required to kill a normal 1 kg American Dutch rabbit. Depending upon the type of experiment, the weights of the rabbits varied from 0.6 to 2 kg. The rabbits were injected on the basis of 1 ml per kg; the maximum dose was 1.0 ml since the susceptibility of a 2 kg rabbit was about the same as that of a 1 kg animal. *Reduced culture filtrate of group A streptococci.* A type 3, D-58 strain was grown for 16 hours in Todd-Hewitt media and then reduced and stored as described by Todd(5). It was titered for streptolysin O activity using standard anti-streptolysin O. Two preparations of this filtrate were used in the work reported in this paper. They are referred to as reduced streptococcal culture filtrate I or II. The streptolysin O titer of both lots was 12.5 combining units per ml. *Normal skin extract.* Sterile broth was injected intracutaneously in place of the streptococcal culture. The skin was then treated exactly as for the preparation of streptococcal lesion extract with the exception that after grinding and the addition of saline it was then subjected to sonic disintegration for 30 minutes in a Raytheon 9 KC oscillator. *Turpentine lesion extract.* This preparation was made exactly as was the streptococcal lesion extract with the exception that 15.0 ml of sterile wood turpentine was injected intracutaneously in place of the streptococcal culture. *Potentiation of toxins and production of the generalized Shwartzman reaction.* American Dutch rabbits weighing about 1 kg were used. The preparing material was injected intradermally or intravenously. After an interval of 4 to 16 hours the provoking injection was given intravenously.

Results. I. Potentiation of lethal effect of typhoid toxin by intradermal injection of streptococcal lesion extract. To determine if products of *in vivo*-grown streptococci could prepare rabbits for the local Shwartzman reaction, the following experiment was performed: 16 rabbits were injected intradermally with 0.5 ml of lesion extract 108 or 109. Sixteen hours later "typhoid toxin" was injected intravenously. As controls, 8 rabbits were injected intradermally with 0.5 ml of "typhoid toxin" and after an interval of 16 hours were injected intravenously with 1.0 ml of the same material. Another control group of 36 rabbits was given a single intravenous injection of 1.0 ml of "typhoid toxin." The results of this experiment are illustrated in Table I. No local lesions were produced in the 16 rabbits receiving lesion extract intradermally and "typhoid toxin" intravenously. However, 13 of the 16 animals in this group died within 24 hours after receiving the injection of "typhoid toxin." Local lesions characteristic of the Shwartzman reaction developed in 50% of the control animals receiving "typhoid toxin" intradermally and the same material intravenously. None of these animals died. Two of the control animals receiving only one intravenous injection of typhoid toxin died. These observations indicated that the intradermal injection of lesion extract in the amount used did not prepare the skin site for the local Shwartzman reaction. However, such an injection did appear to bring about a marked alteration in the general susceptibility of the animals to the intravenous injection of "typhoid toxin" possibly comparable to the preparation of an animal for the generalized Shwartzman reaction.

TABLE I. Potentiation of Lethal Effect of "Typhoid Toxin" by Intradermal Injection of Streptococcal Lesion Extract.*

Procedure	No. of rabbits	No. showing local lesions	No. dead*
0.5 ml lesion extract I.D., "typhoid toxin" I.V. 16 hr later	16	0	13
0.5 ml "typhoid toxin" I.D., "typhoid toxin" I.V. 16 hr later	8	4	0
Single inj. of "typhoid toxin"	36	0	2

* Animals observed for 72 hr. Deaths occurred within 4-24 hr.

II. *Preparation of animals for generalized Shwartzman reaction by intravenous injection of streptococcal lesion extract.* Forty rabbits were prepared with an intravenous injection of lesion extract 109 or 116. After an interval of 4 hours "typhoid toxin" was injected intra-

TABLE II. Preparation of Rabbits for Generalized Shwartzman Reaction by Intravenous Injection of Streptococcal Lesion Extracts.

Preparative material*		No. of rabbits	No. dead†	Microscopic lesions
ml				
.5	lesion extract I.V.	40	35	6 animals which survived over 8 hr studied—4 showed heart lesions, 1 showed renal lesions
.5	normal skin extract	4	0	3 animals studied—0 showed lesions
.5	turpentine lesion extract	4	0	2 " " " " "
2.0	reduced filtrate of 24 hr broth culture of streptococci	6	0	5 " " " " "

* All rabbits inj. intrav. after 4 to 16 hr with "typhoid toxin" as the provocative dose.
† Rabbits were observed 72 hr. All deaths occurred within 28 hr.

venously. As controls for this experiment the following groups of animals were studied: 4 animals were injected intravenously with 0.5 ml of normal skin extract as the preparative injection; 4 animals were treated in a similar manner using 0.5 ml of turpentine lesion extract as the preparative injection; 6 rabbits were prepared with an intravenous injection of reduced filtrate of a 24-hour broth culture of group A streptococci containing 12.5 combining units of streptolysin O per ml. All of these animals were injected intravenously 4 hours after the preparative dose with 1.0 ml "typhoid toxin" as the provocative material. Table II summarizes these observations. Of the 40 animals receiving streptococcal lesion extract as the preparative material, 35 died within a period of 24 hours. In striking contrast, none of the animals receiving normal skin extract, turpentine skin lesion extract, or a filtrate of a 24-hour broth culture of group A streptococci as the preparative material died. These studies indicate that the intravenous injection of streptococcal skin lesion extract markedly alters the susceptibility of rabbits to the lethal effect of the "typhoid toxin." It seems likely that the lethal effect produced involved the mechanisms of the generalized Shwartzman reaction. This possibility was supported by microscopic studies of the hearts and kidneys of rabbits treated in this manner; these animals died or were sacrificed at various intervals up to 72 hours after the injection of the provocative material. The results of these studies are illustrated in Table II. The lesions observed were limited to the animals prepared with streptococcal skin lesion extract. The *lesions in the hearts* consisted of scattered areas in which the myofibers had undergone necrosis. Depending upon the duration of the process at the time of study various degrees of cellular reaction were seen in the areas of necrosis. Between 48 and 72 hours many mononuclear cells with basophilic cytoplasm were seen along with multinucleated giant cells. The myocardial necrosis appeared to be the same as that described by Thomas *et al.*(1-3). No lesions comparable to those described by these workers as fibrinoid necrosis of the coronary arteries were noted. Microscopic studies on control animals receiving only one intravenous injection of "typhoid toxin" revealed no lesions in any of the tissues studied. Sections from one rabbit given sufficient "typhoid toxin" to kill the animal in about 18 hours displayed none of the lesions noted above and showed only areas of hemorrhage in the lung and liver.

III. *Production of generalized Shwartzman reaction using group A streptococcal products as preparative and provocative material.* The work of Shwartzman(6) indicates that it is difficult to obtain a factor from streptococci capable of preparing for the Shwartzman reaction, but a provoking factor is produced. If such a factor could be obtained, it should be possible to bring about the generalized Shwartzman reaction using products derived entirely from group A streptococci. The following experiments were carried out to determine whether such a factor is produced. Groups of rabbits were prepared by the intravenous injection of streptococcal lesion extract 109 or 116. After an interval of 4 hours,

TABLE III. Production of Generalized Shwartzman Reaction Using Group A Streptococcal Products as the Preparative and the Provocative Material.*

Preparative material	Provocative material	No. of rabbits	No. dead†
Streptococcal lesion extr. 109 or 116	Reduced streptococcal culture filtrates	16	13
Single I.V. inj. of 2 ml reduced streptococcal culture filtrate		12	0
Lesion extr. 109	1 ml conc. lesion extr. 112	4	0
" " "	1 ml lesion extr. 109	4	0
" " "	3 ml filtrate of 24 hr culture of type 28 streptococci	6	0
Streptococcal lesion extr. 116	2 ml reduced sterile broth	9	0

* Preparative and provocative materials given I.V. with an interval of 4 hr.
† Animals observed for 72 hr.

TABLE IV. Incidence of Myocardial and Renal Lesions in Rabbits Prepared with an Intravenous Injection of Streptococcal Skin Lesion Extract and Provoked with Various Streptococcal Products.

Provocative material*	Time of death or sacrifice, hr	No. of animals studied	No. showing myocardial lesions	No. showing renal lesions
Reduced streptococcal culture filtrate	24–72	15	11	3
Single I.V. inj. of reduced streptococcal culture filtrate	72	2	0	0
Streptococcal lesion extr. 109	72	3	0	0
Reduced sterile broth	72	5	0	0

* Preparative and provocative materials given I.V. with an interval of 4 hr.

the following materials were injected intravenously to determine their provocative properties: 3.0 ml of a filtrate of a 24-hour broth culture of type 28 streptococci; 1.0 ml of concentrated streptococcal lesion extract 112; 1.0 ml streptococcal lesion extract 109 and 2.0 ml of reduced streptococcal culture filtrate with a titer of 12.5 hemolytic units per ml of streptolysin O. Twelve control rabbits were given 2.0 ml of the reduced streptococcal filtrate without the preparative injection. Another control group was prepared with the lesion extract as described above and after 4 hours received 2 to 4 ml of sterile, reduced Todd-Hewitt broth as a provoking dose. Table III illustrates these observations. Using death as an indication of the generalized Shwartzman reaction, only the material containing active streptolysin O was capable of provoking this reaction. *Microscopic studies* of the tissues also revealed that only the material containing active streptolysin O produced lesions characteristic of the generalized reaction. The results of the microscopic studies are summarized in Table IV. The heart lesions indicated in the table are the same as those described above and appear to be the same as those described as myofiber necrosis by Thomas *et al.*(1-3). These lesions are illustrated in Fig. 1 and 2. No lesion resembling fibrinoid necrosis of the coronary arteries (1-3) was seen in this group of animals. Three of the animals in which the reaction was provoked with filtrate containing active streptolysin O showed characteristic cortical necrosis of the kidney. There was widespread coagulative necrosis of the tissues of the cortex. In many of the glomeruli the capillaries were occluded by hyaline thrombi. These lesions are illustrated in Fig. 3 and 4.

IV. *Immunization of animals against preparatory factor in streptococcal lesion extract.* Rabbits were given one intravenous injection and 3 to 5 intradermal injections of 0.5 ml of streptococcal lesion extract 109 or 116 at weekly intervals. Seven to 10 days after the last injection the animals were given a preparative dose of the lesion extract intravenously

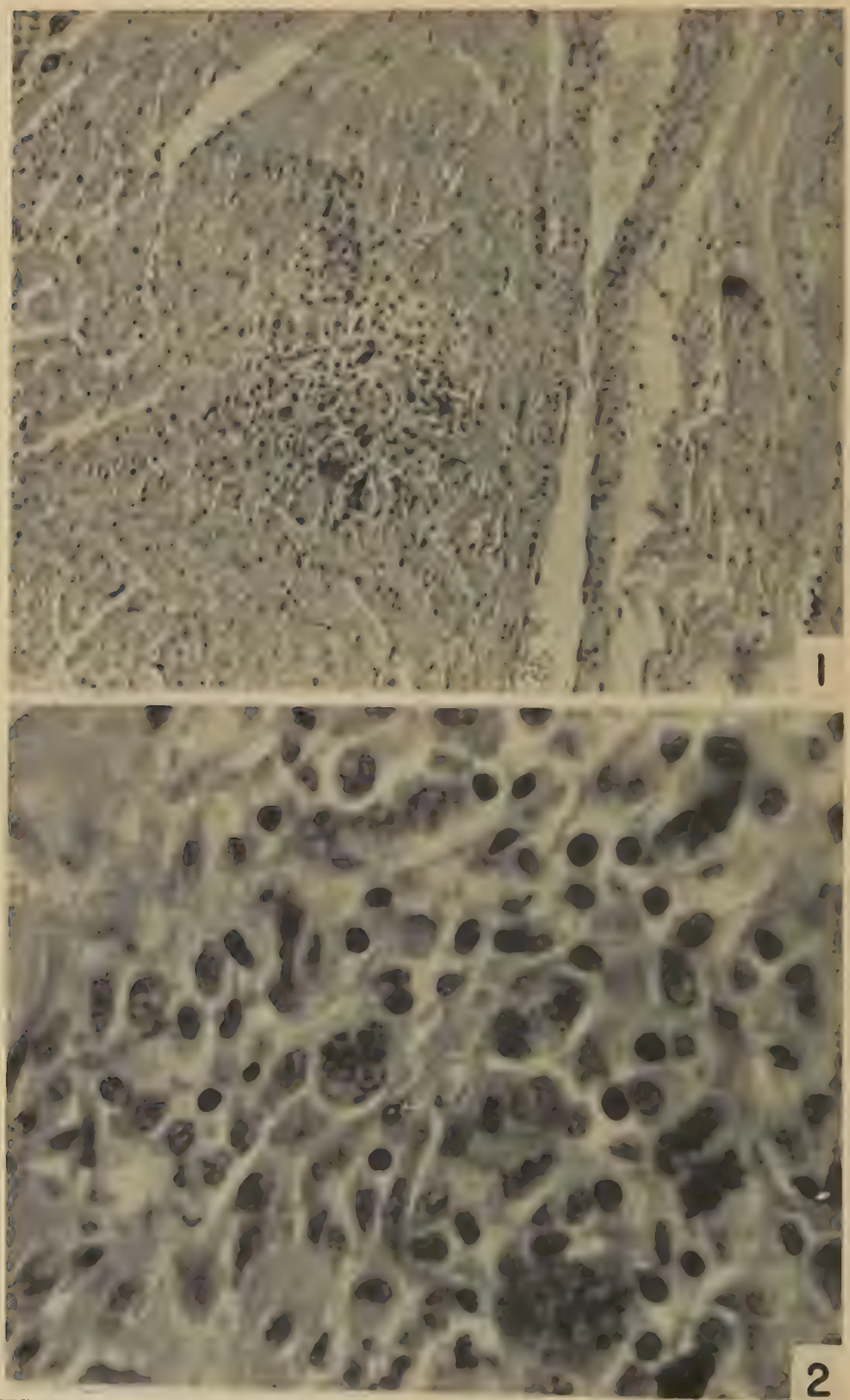

FIG. 1. Heart: Rabbit prepared with 0.5 ml skin lesion extract intravenously. Reaction provoked with 2 ml reduced filtrate broth culture of streptococci which contained 12.5 containing units of streptolysin O. Sacrificed after 72 hr. Focal necrosis of cardiac muscle with associated inflammatory reaction. Hematoxylin and eosin stain. × 200.

FIG. 2. Higher magnification of the lesion shown in Fig. 1. Mononuclear cells and giant cells present in the area of focal necrosis. Hematoxylin and eosin stain. × 900.

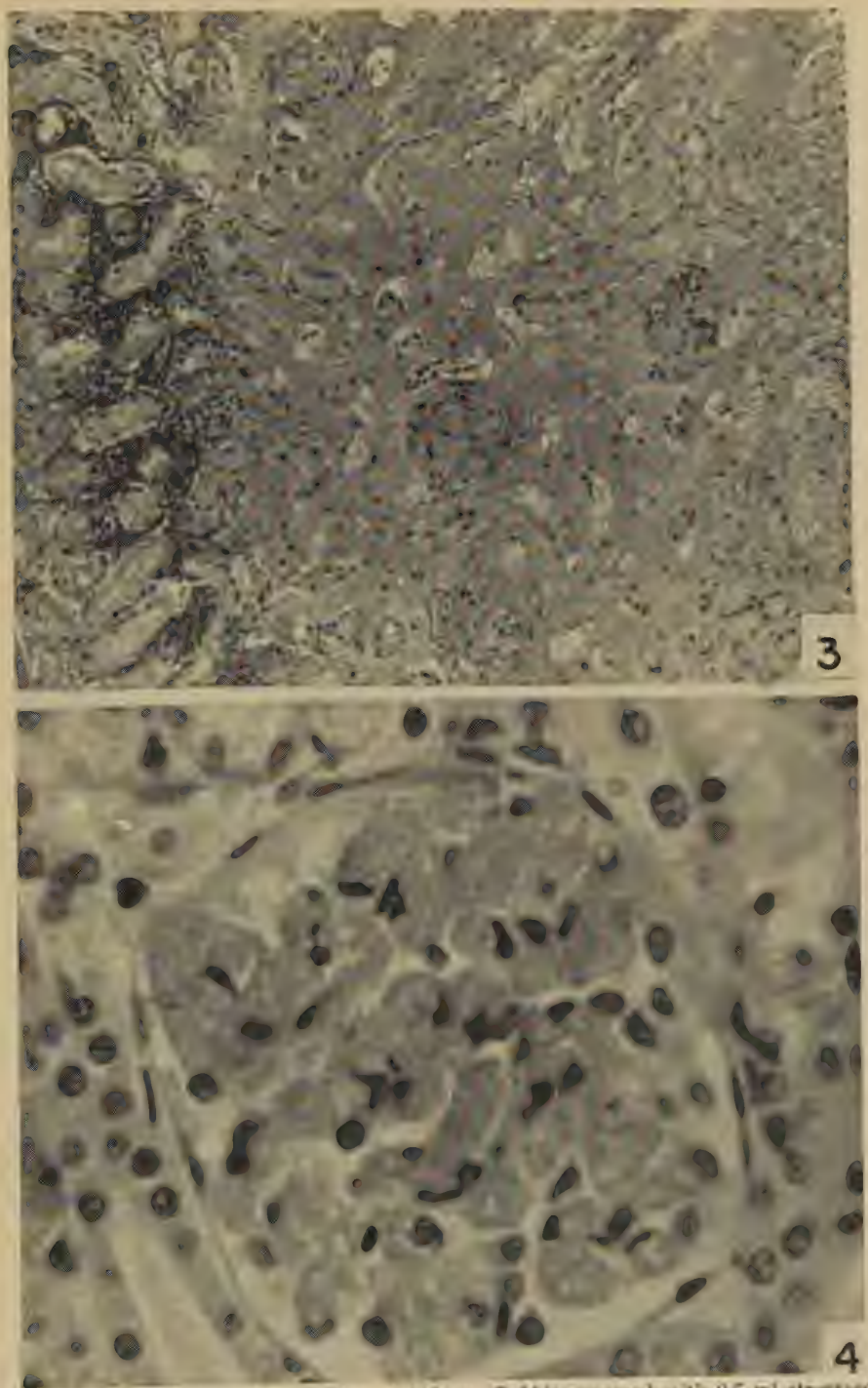

FIG. 3. Photomicrograph of section of kidney. Rabbit prepared with 0.5 ml streptococcal lesion extract intravenously. Reaction provoked by intrav. inj. of 2 ml *reduced* filtrate of broth culture of streptococci containing 12.5 combining units of streptolysin *o*. Animal died after 19 hr. Cortex shows diffuse necrosis with an associated zone of cellular infiltration beneath the capsule. Hematoxylin and eosin stain. × 200.

FIG. 4. Photomicrograph of glomerulus. Reaction produced in same manner as described above for Fig. 1. Animal died at 8 hr. Glomerular capillaries are filled with a homogeneous acellular material. Hematoxylin and eosin stain. × 900.

TABLE V. Immunization of Rabbits against the Preparative Factor in Streptococcal Lesion Extracts.*

Exp. No.	Immunized	Control
1	1/6†	4/5†
2	0/9	8/8
3	1/6	6/7
Total	2/21	18/20

* Rabbits prepared with homologous lesion extr. followed after 4 hr by "typhoid toxin."
† Dead/total.

and 4 hours later received a provocative dose of "typhoid toxin." The non-immunized control rabbits received the same preparative and provocative injections. The results of 3 such experiments are shown in Table V. A total of 18 of 20 control rabbits died in contrast to 2 out of 21 in the protected group. These results indicate that the rabbits can be immunized against the preparative material present in the lesion extract.

Discussion. It has been shown that the intradermal or intravenous injection of a soluble material produced when group A streptococci multiply *in vivo* possesses the property of increasing the susceptibility of rabbits to the lethal effect of filtrates of the Gram-negative organism, *Salmonella typhosa*. It is believed that this increased lethal effect involves the mechanisms of the generalized Shwartzman reaction. However, it should be pointed out that there are several differences between the phenomenon observed in this paper and the classical Shwartzman reaction. In our experiments no local reaction has been observed upon intradermal preparation with the whole lesion extract, but the generalized reaction is produced by this preparing route. It is possible that the hyaluronidase which is present in the lesion extract may allow a more rapid diffusion of the active factor into the circulation, thus giving the effect of an intravenous preparative dose. The observation of death of the animals prepared with the lesion extract is definitely more prominent than is usually observed in the generalized Shwartzman reaction. There is also a reduced incidence of renal necrosis which is usually characteristic of the generalized reaction. However, the cardiac lesions recorded in this paper are similar to those reported by Thomas *et al.*(1-3) as typical of a generalized Shwartzman reaction. Finally, the time interval between the preparative and provocative injections can apparently be reduced to a much shorter time in our experiments than is possible in the classical reaction described by Shwartzman(6). Of primary significance is the observation that reactions in rabbits can be provoked with reduced filtrates of streptococcal cultures. Filtrates in which the streptolysin O activity was maintained appeared to be the most effective provocative material under the conditions of these experiments. The fact that streptolysin O is the active factor in the provocative injection is difficult to prove without having a pure product with which to work. Further studies to clarify this point are being carried out at the present time.

The fact that rabbits can be prepared and provoked for the generalized Shwartzman reaction with factors derived entirely from group A streptococci adds significance to the possible role of this reaction in the pathogenesis of the nonsuppurative sequellae associated with group A streptococcal infections.

The most common lesions observed were found in the heart; however, renal cortical necroses were noted in a number of animals prepared with skin lesion extract and provoked with culture filtrate in which the streptolysin O activity was maintained. The cardiac lesions were similar to those referred to by Thomas *et al.*(1-3) as myofiber necrosis. The fibrinoid necrosis observed in the arteries of the heart by Thomas *et al.* was not observed in these studies.

It is of interest to note the small quantity of the active factor in the lesion extract which is required to prepare the animals. The protein concentration of the extract, based on total nitrogen, is approximately 2%. Electrophoretic studies indicate that most of this is rabbit serum protein. Thus, the 0.25 ml volume injected must contain a minute amount of the active material. The *nature of the resistance* of the rabbits immunized to the preparing factor is not known. It is probably not the same as that developed against bacterial pyrogens as described by Morgan(7), and Beeson (8,9). Studies, not yet complete, indicate that the resistance described in this paper is type

specific. The *nature of the soluble material* extracted from group A streptococcal skin lesions which is responsible for preparing animals for the generalized Shwartzman reaction is being investigated. The precise relationship of this product and the provoking factor in the reduced culture filtrate to the generalized Shwartzman reaction is under study.

Summary. A soluble factor from group A streptococcal skin lesions prepared American Dutch rabbits for the generalized Shwartzman reaction. The reaction can be provoked in rabbits prepared with this material not only with toxins from Gram-negative organisms but also by reduced filtrates of streptococcal cultures with a high streptolysin O content. The tissue changes associated with the reaction brought about with these materials include myocardial necrosis and cortical necrosis of the kidney. Although these reactions simulate those observed in the classical generalized Shwartzman reaction, points at difference are discussed. It was possible to immunize rabbits against the preparative activity of the soluble factor by repeated injections of the streptococcal skin lesion extract.

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Renal Function During Viomycin* Administration to Dogs. (20238)

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An intermittent dosage schedule for administering Viomycin (2 g twice a week) to patients with pulmonary tuberculosis, proved to be free of nephrotoxicity(1). It is not apparent whether this absence of toxicity is due to administering the antibiotic intermittently or due to smaller doses than those employed in the original studies when nephrotoxicity(2,3) was observed. Therefore, before giving larger doses of Viomycin to patients, a preliminary study of nephrotoxicity and electrolyte disturbances using larger daily doses (15 to 60 mg/kg/day) in dogs seemed indicated. The purpose of the present communication is to report these observations.

Methods. Six female dogs were studied. The dogs were observed for a control period of two weeks during which time renal function (clearance) and electrolyte (plasma) studies were completed. Then they were placed on a Viomycin regimen receiving one-half the daily dose intramuscularly in the morning and one-half in the evening. Three of the dogs received a dose of 15 mg/kg for 3 months which was then increased to 30 mg/kg for an additional 3 months. Three of the dogs were started on 30 mg/kg. After 3 months this was increased to 60 mg/kg which they received for an additional 3 months. After the drug was started, renal function studies were repeated once a month as long as Viomycin was being administered. The dogs were weighed daily throughout the study. Creatinine was used to measure glomerular filtration rate, p-aminohippurate (PAH) for renal

* Supplied through the courtesy of Parke-Davis and Co.

TABLE I. Effect of Viomycin on Renal Function in 6 Dogs.

	Dose, mg/kg		Wt, kg			GFR			RPF			TmG		GFR/TmG		RBF		
	D_1	D_2	C	D_1	D_2	C	D_1	D_2	C	D_1	D_2	C	D_2	C	D_2	C	D_1	D_2
	15	30	11.9	13.5	14.9	29	30	33	113	104	91	110	116	.26	.28	177	186	175
	15	30	9.9	10.5	11.3	25	31	30	100	102	84	81	80	.31	.38	167	179	156
	15	30	9.5	9.2	9.1	25	24	23	65	60	62	64	70	.39	.33	81	71	72
	30	60	10.6	11.9	12.5	26	29	30	94	113	68	81	76	.32	.39	159	182	97
	30	60	10.0	10.7	10.0	28	27	32	74	67	69	82	91	.34	.35	119	120	105
	30	60	11.4	11.1	11.6	28	33	40	84	122	115	106	105	.26	.38	153	298	205
Mean	23	45	10.6	11.2	11.6	27	29	31	88	95	82	87	90	.31	.35	143	173	135

C, control; D_1, after receiving Viomycin for 3 mo; D_2, after receiving Viomycin for 6 mo; GFR, glomerular filtration rate in ml/min.; RPF, renal plasma flow in ml/min.; TmG, maximum tubular reabsorption of glucose in mg/min.; RBF, renal blood flow, $\frac{\text{RPF}}{\text{1-Hct.}}$

TABLE II. Effect of Viomycin on Electrolytes and Hematocrit, in 6 Dogs.

	Serum Na*			Serum K*			Na exc.†			K exc.†			Hematocrit		
	C	D_1	D_2	C	D_1	D_2	C	D_1	D_2	C	D_1	D_2	C	D_1	D_2
	135	138	131	3.5	3.3	3.6	.06	.22	.09	.10	.03	.08	36	44	48
	133	139	141	2.7	3.4	3.8	.24	.34	.40	.09	.02	.03	40	43	46
	133	138	135	2.7	3.5	3.1	.09	.21	.05	.07	.04	.07	20	16	14
	136	142	140	3.5	3.4	4.1	.13	.44	.16	.17	.19	.16	41	38	30
	137	144	137	3.0	3.3	3.9	.13	.20	.36	.04	.02	.06	38	44	34
	133	143	141	3.1	2.7	3.4	.07	.65	.76	.02	.17	.23	45	59	44
Mean	135	141	138	3.1	3.3	3.7	.12	.34	.30	.08	.08	.11	37	41	36

C, control; D_1, after receiving Viomycin for 3 mo (see Table I); D_2, after receiving Viomycin for 6 mo.

* mEq/liter. † mEq excreted in urine/min.

plasma flow and dextrose for the maximum reabsorptive capacity of the renal tubules (TmG). The latter determination allowed an evaluation of renal tubular damage. Mean blood pressure was measured by a manometer connected to an indwelling arterial needle. Arterial blood was used for chemical analysis. Serum sodium and potassium concentrations were determined once a month throughout the study. A Beckman flame photometer was used for these analyses. Except for minor modifications, the techniques used have previously been described (4).

Results. The observations on renal function are presented in Table I. There was some variation in glomerular filtration rate and renal blood flow from month to month but this was inconstant and represents normal variation, even while receiving doses as large as 60 mg/kg for 3 months. Three of the dogs actually gained weight during the study.

Viomycin seemed to have very little effect on serum electrolytes. There appeared to be a slight increase in sodium excretion. However, this may have been due to altered sodium intake since it was not increased significantly after the first 3 months of treatment despite the increase in the amount of Viomycin being administered.

Discussion. The freedom from nephrotoxicity in this group of dogs who were being given large daily doses of Viomycin suggests that Viomycin in current use may not be as toxic to the kidney as the earlier studies would indicate provided, of course, that patients are not more sensitive to the antibiotic than are dogs. Although 60 mg/kg may not be a large dose for a dog, this amount of drug given to a 70 kg man would amount to 4.2 g per day. The relatively normal plasma electrolytes during the study while the animals were receiving Viomycin further suggests that the drug does not affect the kidney adversely, particularly the tubular reabsorptive mechanism.

Summary. Daily doses of 15-60 mg/kg of Viomycin administered for a period of 6 months did not appear to produce any evidence of renal toxicity as estimated by renal

function studies (clearances). There were no serious alterations of the concentrations of serum sodium and potassium. These data suggest that the renal toxicity and altered electrolyte metabolism associated with Viomycin therapy may not be as serious as the earlier observations indicated. Therefore, we believe that further use of Viomycin with careful observations for signs of renal toxicity is indicated, particularly in patients unresponsive to the other more effective therapeutic approaches.

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Regeneration of Lens and Retina in Thyroidectomized and Hypophysectomized Adult Newt (*Triturus v. viridescens*).* (20239)

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The senior author(1-4) has been making an experimental study of factors affecting the release and inhibition of lens regeneration from the dorsal iris in adult eyes of the newt, *Triturus v. viridescens*. It has recently been reported by Hall and Schotté(5) that hypophysectomy, and by Richardson(6,7) that thyroidectomy and hypophysectomy markedly affect regeneration of the limb of the newt. In view of this it became essential to know what effect, if any, the removal of these endocrine glands might have on the regeneration of the lens from the dorsal iris as well as the neural retina pigment cells(8) in the newt. Already Nikitenko(9) has claimed that hypophysectomy in adult newts (species?) had no direct effect upon lens regeneration, and Lenhard(10) reported that the lens could regenerate normally in thyroidectomized adult *Triturus cristatus*, although the results were not consistent.

In the present experiments one group of adult newts was hypophysectomized, another was thyroidectomized, and in another both the hypophysis and the thyroids were excised. Four to 8 days later the lens was removed from the right eyes and both the lens and the neural retina were removed from the left eyes. Many animals did not survive the duration of the experiments. Therefore the results here are based on a study of a total of 173 eyes (81 in the first group, 48 in the second and 44 in the third) in animals sacrificed at frequent intervals from 2-43 days after the eye operations. In many specimens in each group the right eyes were later excised in alcohol and cut along the equator to separate the anterior half for gross examination of the relations of the regenerating lens to the iris. These were later prepared in serial sections as were all other eyes. Also serial sections of all heads and jaws were studied to detect whether or not all the thyroid and pituitary tissue had been removed. Similar gross and histological preparations, fixed at frequent intervals from 2-60 days after operation, were examined as normal controls in 178 eyes from which only the lens was removed in order to determine in eyes with intact retina the average, as well as the individual variations, in the rate of lens regeneration and

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growth within similar age groups. For comparison another series of 84 control eyes was analyzed from which both the lens and the neural retina were excised. These were preserved at frequent intervals from 2-57 days after operation. Sato's(11) staging of lens regeneration in adult *Triturus pyrrhogaster* was applied in these studies where it was possible. The room temperature at which the animals were kept was about 70-75°F.

Results. Two of the 24 animals from which only the thyroids were removed, showed accessory thyroid follicles embedded among jaw muscles. Accessory follicles, usually from 1 to 3, were frequently found in animals from which the pituitary was removed. In each of 3 cases in the latter group there was found a small fragment of the adenohypophysis as well as a small remnant of the neurohypophysis. Also in 4 others a fragment of the neurohypophysis had not been removed. However, in those instances where accessory thyroid follicles or a small fragment of the pituitary was left behind, general after effects of the gland operation and the course of lens and retina regeneration appeared to be no different than in the many from which the tissues of the glands in question had been completely removed.

The animals from which the glands were removed were usually thin and not very active. A thick layer of cornified epidermis and mucus covered the body. Some maintained their normal body color but in others the skin became very dark over dorsal and lateral surfaces with spreading of dark layers on the ventral surface as well. Molting became generally very irregular and failed to occur in many of them. In some there were localized dark spots over the head, body and limbs. A characteristic V-shaped spot appeared on the dorsum of the head in a number of hypophysectomized animals.

Following lens removal in adult eyes with the retina intact, there are distinct variations in the rate of lens regeneration within similar age groups, although regeneration in general is steadily progressing. For example, the thickening of the dorsal iris, the proliferation and depigmentation of cells along the pupillary margin are 3 early stages of lens regeneration which overlap within similar age groups from 6-10 days after operation. Various stages in the formation of the lens vesicle and its primary lens fibers also overlap within similar age groups during the second and particularly the third week. Again within age groups during the third week there is an overlapping of various stages covering the rapid differentiation and acquisition of secondary lens fibers. However during the fourth week most lens regenerates have a completely developed lens capsule and usually become detached from the dorsal iris about the twenty-fifth day. As the secondary lens fibers accumulate and rapidly increase the size of the lens, the cell nuclei of the central primary lens fibers are gradually lost by the end of the second month. However when both the lens and the neural retina are removed, the rate of regeneration and the growth of the lens regenerate are much retarded during the first month. During most of the second month when further detailed staging of lens regeneration is not sharply defined the lens size is still distinctly affected, but by the end of the second month it approaches that of the lens regenerates in eyes with intact retinae.

The delay of the early stages of lens regeneration is associated with the progress of neural retina regeneration from the retinal pigment cells, which, after recovery from the operation require about 2 weeks or longer to establish a layer of depigmented cells. When these cells begin rapid proliferation during the third week the dorsal iris gives rise to the lens vesicle. Its growth and differentiation then proceed along with that of the regenerating neural retina. Differentiation in the latter may be well advanced during the fourth week, and complete soon thereafter while the secondary lens fibers are rapidly accumulating in the growing lens regenerate.

Measurements of lenses in animals from which the glands were removed, showed as in the normal controls, that lens regeneration in the left eyes with regenerating retinae was retarded when compared with that in the right eyes with intact retinae. In fact the rate of lens regeneration in both the right and left eyes followed closely that of comparable eyes in the normal controls. Also in the left

eyes of animals from which the glands had been removed the neural retina was slowly regenerating from the pigment cells almost as rapidly as in the normal control left eyes. Advanced differentiation of the retina layers appeared in some at the end of a month and in others they were still more retarded at this time. Eyes preserved later showed complete retina regeneration.

Although there was no marked effect of gland removal upon the rate of lens regeneration there was a definite effect upon the size of the lens regenerates. Where they could be measured they were definitely smaller than the normal controls, indicating that proliferation in the lens regenerates was at a slower rate. Possibly associated with this is the fact that various stages of cataract formation were very frequently found. This was observed in various groups from 18-50% of the stages in which the primary fibers at least are fully differentiated (Sato's stages 9 and above). Malformed lenses and lentoids were also observed in them. Such abnormal lenses seldom appeared in normal control eyes after plain lens removal. In the left eyes removal of the retina may have played some role, for in the normal control groups from which both the lens and neural retina were removed cataracts were often found (23.4%).

Summary. 1. Lensectomy only and lensectomy combined with retina removal was performed in adult newts 4-8 days after the excision of the thyroids, the hypophysis or both. The results were compared with those in control experiments. Normally lens regenerates reach full shape and structure about the eighth week when cell nuclei of the primary fibers disappear. Rate of regeneration as well as growth of lens were distinctly diminished after retina removal. Early stages of retina regeneration proceeded for about 2 weeks before a lens vesicle was formed. Retina differentiation might be completed during the fifth week. 2. Measurements of lenses showed that regenerates developing in the presence of a regenerating retina were much behind those in eyes with the retina intact. Development rate in both kinds of eyes was very similar when eyes of normal and of gland-deprived animals were compared. In the latter, retina regeneration was not significantly retarded. The size of the lens regenerates at various dates after the eye operation was considerably less in gland-deprived animals. Gland removal seemed to increase the incidence of malformed regenerates and of cataracts. The incidence of cataract was also increased in normal controls after removal of the retina.

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Virulence of *Coccidioides immitis* Determined By Intracerebral Inoculation in Mice. (20240)

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The low virulence of pathogenic fungi for laboratory animals (as expressed by death rates) is well known. Several authors have investigated the virulence of different fungi, and sought to establish reasonably constant death rates. They accomplished this either by decreasing the resistance of the animals by means of X ray(1), or by incorporating the inoculum in mucin(2-5), by using the intracerebral route of inoculation(6,7), or by using a more susceptible mouse strain(7). But no attempt was made in these studies to calculate the LD_{50} and to compare the virulence of different fungus strains.

For many reasons, it was felt desirable to investigate methods which would measure and compare the virulence of strains of *Coccidioides immitis*. Since death rates are a convenient and simple measure for virulence, computation of the LD_{50} was considered the primary purpose of such work. On the basis of the established LD_{50} the virulence of different strains would then be comparable. Since *C. immitis* inoculated intraperitoneally causes death in mice only if rather concentrated inocula are used, this was judged to be an unsatisfactory way for computing and comparing death rates. Of the virulence enhancing methods the most promising one seemed to be the intracerebral route of inoculation, employed by Howell and Kipkie(6) for obtaining death rates with *Histoplasma capsulatum* in mice. Although pigmented mice were found more susceptible to *Histoplasma*(7) and to *Blastomyces dermatitidis* (8) than albino mice, the former variety seemed less practical because pigmented mice are difficult to obtain, and more expensive. Therefore, the response of albino mice to intracerebral inoculation with *C. immitis* was made the subject of this investigation.

Materials and methods. Two strains of *C. immitis* were used: Strain No. 973 (Tucson, Ariz., 2-13-1946) was isolated from sputum; strain No. 2226 (Tampa, Fla., 6-1-1950) was isolated from a draining sinus. Old cultures of these strains, grown on Sabouraud's glucose agar at room temperature, were examined microscopically for the presence of arthrospores and saline suspensions made without the aid of a dispersing agent. Ten-fold dilutions in saline were made of the original suspension, and 0.2 ml aliquots of each dilution was streaked on Sabouraud's glucose agar plates (5 or more plates for each dilution). Such plates were incubated at 37°C and colony counts made after 2 to 4 days. Depending upon the number of colonies per dilution, 4-fold dilutions of the original suspension were made so that they contained a number estimated to be between 0 and 100 fungus particles per intracerebral inoculum (0.03 ml for young mice, 0.05 ml for old mice). Locally bred albino mice of different ages and both sexes were used in the experiment. Although no difference of susceptibility was noted in different age groups, animals of approximately the same size and age were used in each experiment. The mice were inoculated into the left cerebral hemisphere under anesthesia, usually 5 to 6 animals per dilution, using several 4- or 5-fold dilutions. LD_{50} estimates were obtained by the method of Reed and Muench(9), and the Standard Error $\log \cdot LD_{50}$ was computed as proposed by Pizzi (10). Sabouraud's glucose agar plates were inoculated, using the same syringes and dilutions with the amount of suspension injected into each mouse (0.03 ml or 0.05 ml). Sterile saline (0.2 ml) was added to each plate and the inoculum streaked with a sterile glass rod to insure even distribution of particles. Colony counts were made after the plates were incubated at 37°C for 2 to 4 days and again 1-2 days later. Although the counts were sometimes higher on the second examination, other times they were decreased because larger colonies had fused together. The higher counts were considered final and were used to calculate the regression line (method of least

TABLE I. Statistical Data on Regression and Fiducial Limits of LD_{50} for 2 Strains of *C. immitis*.

Strain	a*	b*	$s_{y \cdot x}{}^2$†	y‡ LD_{50}	y‡ LD_{50}+2SE	y‡ LD_{50}−2SE	$s_y \cdot t_{95\,pc}$§ LD_{50}+2SE	$s_y \cdot t_{95\,pc}$§ LD_{50}−2SE	95% fid. limits of yLD_{50}
973	.25	.12	2.71	1.2	2.7	.6	3.57	3.65	1 to 6.3
2226	1.29	.21	5.35	3.0	3.9	2.4	4.98	5.02	1 to 8.9

* Parameters of regression. † Square of stand. error of the estimate. ‡ Plate count, estimated from regression, corresponding to LD_{50}, LD_{50}+2 S.E. LD_{50}, and LD_{50} − 2 S.E. LD_{50}. § pc = %.

TABLE II. Experimental Data of Intracerebral Titration of 2 Strains of *C. immitis* in Albino Mice.

Strain	Dilution by vol.	Mice*	Survival of mice (days after inoc.)	Plate counts
973	1: 64000	3/3	14, 15, 15	11, 9, 8, 3
	1: 256000	2/5	14, 19	5, 3, 2, 2, 1
	1:1024000	3/5	12, 17, 18	1, 1, 1, 1, 0
	1:4096000	0/3		0, 0, 0, 0, 0
	LD_{50}† = 1:512000.			
	S.E. log LD_{50}‡ = ±0.200.			
	Probable No. of particles§ representing LD_{50} = 1 to 6.			
2226	1: 1600	5/5	10, 14, 15, 15, 16	19, 16, 14, 13, 10
	1: 6400	4/5	12, 15, 16, 18	9, 8, 6, 6, 4
	1: 25600	1/5	14	3, 2, 1, 0, 0
	1: 102400	0/5		2, 1, 1, 0, 0
	LD_{50}† = 1:12800.			
	S.E. log LD_{50}‡ = ±0.095.			
	Probable No. of particles§ representing LD_{50} = 1 to 9.			

* Numerator = dead mice, denominator = total No. of mice.
† Method Reed and Muench(9). ‡ Method Pizzi(10). § From Table I, last column.

squares). On the abscissa the doses were plotted, using code numbers 1, 4, 16, and 64 instead of the dilution fractions. On the ordinate the corresponding plate counts were plotted. The count y corresponding to LD_{50} ± 2 S.E. LD_{50} was estimated from the formula of the line, and the corresponding $s_y{}^2$ calculated.* Fiducial 95% limits of plate counts (y) corresponding to LD_{50} ± 2 S.E. LD_{50} were then determined.† The 95% fiducial limits of the plate count representing LD_{50} were considered to be not less than 1, and to lie between $y_{(LD_{50} - 2\,S.E.\,LD_{50})} - s_y \cdot t_{95\,per\,cent}$ and $y_{(LD_{50} + 2\,S.E.\,LD_{50})} + s_y \cdot t_{95\,per\,cent}$. The statistical data as calculated from the experimental data of Table II are summarized in Table I.

Results. The experimental data of 2 titrations are summarized in Table II. It will be seen in this table that each strain had a high degree of virulence, *i.e.*, the LD_{50} was found to correspond to 1 to 6 and 1 to 9 fungus particles for strains No. 973 and No. 2226, respectively.

The survival time of inoculated mice increased with decreasing doses but the animals succumbed well within the 30-day period established by Howell and Kipkie(6) for *H. capsulatum*. With doses below 100 fungus particles the survival time ranged between 10 and 20 days. Where larger doses ranging from 1:400 to 1:1600 by volume were used (experiment not recorded in this paper) death occurred in as few as 3 days.

Early signs of infection included ruffled hair, hunched back and an attempt to hide the head. Later symptoms included the whole scale of nervous signs such as partial or complete paralysis, tonic and clonic convulsions, and vestibular disturbances. Frequently, a bulging of the parietal skull suggested increased intracranial pressure. No animal showing clinical symptoms of infection survived the experiment.

Cultures of the brain were made on Sabou-

* $s_y{}^2 = s_{y \cdot x}{}^2 \cdot \left(1 + \frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma (x-\bar{x})^2}\right)$.

† $y \pm s_y \cdot t_{95\,per\,cent}$

raud's glucose agar at the time of death. All cultures were positive and sections made from a small number of specimens showed the tissue phase (spherule) of *C. immitis* within the brain substance and the meninges. Occasionally, a localized abscess was seen over the bone of the left hemisphere which corresponded to the inoculation site. Such areas also were positive on culture.

Cultures of the brains of some of the *surviving* mice were negative even though the entire left half of the brain was used as inoculum. Other surviving mice were reinoculated intracerebrally with a massive dose of the fungus. All such mice died with typical symptoms.

Discussion. Ophuls(11) and Stewart and Meyer(12) suggested that mycelial fragments devoid of arthrospores obtained from cultures of *C. immitis* were non-infectious. Since both mycelial fragments and arthrospores may germinate to give colony counts of a suspension made from a culture of this fungus, such a colony count may not be indicative of the exact number of infectious particles present in the suspension. Therefore, although the 2 strains of *C. immitis* used in the present investigation were known to produce abundant arthrospores in old cultures, the exact number of infectious particles (arthrospores) injected in each dose may not have corresponded exactly with the plate counts.

The experiments have shown, however, that large colony counts from suspensions were not necessary to produce symptoms and death when such suspensions were inoculated intracerebrally into mice. Rather, the LD_{50} and MLD counts were found to be of such a low order as to suggest that the prepared inoculum contained a majority of infectious arthrospores with few non-infectious mycelial fragments.

The results of the above experiments do not permit the conclusion that there is a real difference of virulence in strains No. 973 and No. 2226, at least if the LD_{50} is estimated with 95% fiducial limits. In these experiments, however, only a few dilutions of inoculum, and small numbers of mice and plates were used. If these were to be increased, a statistical evaluation would give more narrow limits for the LD_{50} whereby small differences in virulence could be detected.

The intracerebral route of infection, therefore, seems to provide an useful means for comparing the virulence of different strains of *C. immitis*.

Summary. The virulence of 2 strains of *Coccidioides immitis* was evaluated by intracerebral inoculation in albino mice. The LD_{50} for the 2 strains was found to be between 1 to 6 and 1 to 9 fungus particles.

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Antituberculous Activity of Substituted Thioureas. (20241)

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Mycobacteria, actinomycetes and streptomycetes are members of the order Actinomycetales and are characterized as organisms forming elongated cells which have a definite tendency to branch. This morphological feature places the members of this order close to the fungi taxonomically. In an earlier study(1) one of us (R.L.M.) investigated the hypothesis that this morphologic relationship between mycobacteria and fungi may be paralleled by a biochemical relationship and thereby suggesting a basis for the search for new antituberculosis agents. It was indeed found that a number of recognized antifungal substances exerted strong and specific antimycobacterial *in vitro* activities while possessing only minor effectiveness against the common Gram negative and Gram positive bacteria. Particularly interesting among the sulfur-containing compounds exhibiting such properties were thiourea and its derivatives. The most active substance was sulfamidothiourea (2255 RP).

We have now extended these earlier studies and investigated more than 350 thiourea derivatives and related compounds for their antimycobacterial and antifungal activities. Many of the disubstituted thioureas, especially the thiocarbanilides demonstrated not only *in vitro* tuberculostatic and fungistatic activities but were also found to have excellent chemotherapeutic effects in mice and guinea pigs infected with the tubercle bacillus. From among the many thiocarbanilides studied, several have been selected for inclusion in this paper as representative of the various types of substituents located in the p,p'positions (Table II).

In vitro activities of disubstituted thioureas. Numerous derivatives demonstrated considerable activity against the human, bovine and avian types of the tubercle bacillus as well as the BCG (human) strain. However, none of these compounds showed appreciable activity against the saprophytic mycobacteria or any of the usual Gram negative or Gram positive bacteria. Furthermore, a number of compounds simultaneously exhibited excellent activities against the tubercle bacillus and a variety of fungi, as evidenced by the examples shown in Table I.

Chemotherapeutic activities in mice. Experiments with representative types of thioureas. CF1 mice, weighing between 15 and 20 g each, were infected intravenously with 0.5 ml of a 1:10 dilution of culture of $H_{37}Rv$ (or Ravenel) grown for 7 days in Kirchner's liquid medium containing 0.05% Triton A-20 and 0.5% bovine serum albumin. Immediately upon infection, groups of 10 mice each were fed for 21 days with a diet consisting of ground food mixed with finely pulverized (200 mesh) test compounds. Normal diet was restored for an additional fifteen days and all surviving animals then sacrificed. All active thiourea derivatives were tested at varying concentrations and the percentages indicated in Table II represent the lowest dose levels given to the infected groups of animals. *T50* represents the calculated survival time (days) of 50% of the mice and is obtained by plotting the cumulative percentage dead on a probability scale against time on an arithmetic scale(3). Since the average T50 for the infected, untreated groups was 20 days in this experiment, values greater than 23 days are indicative of chemotherapeutic activity.

The results obtained in the diet experiments with 11 selected substituted thioureas bearing a variety of substitutions in the p,p'-positions are shown in Table II. From these results it is readily apparent that the presence of certain substituents, such as alkoxy groups, in p,p'positions either with or without nitrogen, or alkyl groups, has transformed the inactive unsubstituted thiocarbanilide (Su-1495/E) into substances with considerable antitubercular activity in mice.

Additional experiments with Su 1795. The extent of activity of the disubstituted thiou-

TABLE I. *In Vitro* Activities of Selected Thioureas.

	Substance		Su 1814	Su 1380	Su 1515	Su 1795
Structure:		R	$CH_3CH_2CH_2CH_2-$	CH_3CH_2O-	$CH_3CH_2CH_2CH_2O-$	CH_3CH_2O-
	R-⟨⟩-NHCSNH-⟨⟩-R_1	R_1	$-CH_2CH_2CH_2CH_3$	$-OCH_2CH_3$	$-OCH_2CH_2CH_2CH_3$	$-OCH_2CH(CH_3)_2$
I	Mycobacterium tuberculosis, $H_{37}Rv$	tube*	1:1000000	1:250000	1:1000000	1:1000000
II	*Trichophyton interdigitale*	tube	1: 50000	1: 1000	1: 1000	1: 10000
		disc*		>90 mm		>90 mm
	" *rubrum*	tube	1: 50000	1: 1000	1: 1000	1: 10000
		disc		>90 mm		>90 mm
	Candida albicans	tube	1: 50000			<1: 1000
		disc		>90 mm		
	Torula lipofera	tube		1: 1000		1: 10000
		disc		>90 mm		
III	*Staphylococcus aureus*	tube	<1:500	<1:500	<1:500	<1:500
	Streptococcus pyogenes	tube	"	"	"	"
	Escherichia coli	tube	"	"	"	"
	Salmonella schottmüelleri	tube	"	"	"	"
	Antitubercular activity *in vivo* (mice & guinea pigs)		active	active	active	active

*"Tube": refers to tube-dilution broth method; values shown are highest dilutions completely inhibiting growth of organism. "Disc": indicates diameter of zone of inhibition obtained on Sabouraud's agar by the agar-disc method, using 1/50 molar conc.

reas, as represented by Su 1795, is evident from the following additional experiments.

a) *Delayed therapy.* As is shown in Table III, considerable therapeutic activity is obtained when diet-therapy with 0.5% Su 1795 is delayed for as long as 15 days or with 0.1% for 9 days.

b) *Limited therapy.* According to the data presented in Table IV, a total treatment period of only 6 days with a diet containing 0.1% Su 1795 or only 12 days with 0.05% resulted in effective antituberculosis action.

Chemotherapeutic activity in guinea pigs; Experiment Bk 5/214. Procedure: Female guinea pigs weighing approximately 600 g were each infected subcutaneously with 1.0 ml of a 1:200 dilution of a 7-day-old culture of $H_{37}Rv$ in the modified Kirchner's liquid medium. Twenty-one days after infection, the tuberculin-positive animals were treated with medicated diets. Therapy was maintained for the following 115 days and the animals then sacrificed. The evaluation of the gross organ involvement was based on the method described by Feldman(4) who assigned maximum values of 40, 70 and 100 for slight, moderate and extensive degrees of total organ involvement respectively. In Table V are reported results obtained with 5 different thiourea-derivatives; 4 of the 5 substances possessed good tuberculostatic activities in guinea pigs at doses as low as 0.05% in the diet.

Experiment Bk 12/1. In a delayed therapy experiment with guinea pigs, similar to the one performed with mice (Table IV), diet-therapy with Su 1795 was started 60 days after infection and continued for 100 days. The results are given in Table VI and Fig. 1 which is a schematic representation showing the extent of tuberculous involvement for each animal at time of necropsy(5). According to this data, Su 1795 compares favorably with Isoniazid at the same dose level in exerting a therapeutic effect in a well-established guinea pig infection.

Discussion. In an extension of earlier studies(1) on antifungal and antimycobacterial agents, a large number of synthetic sulfur-containing compounds, particularly thiourea derivatives, have been investigated. Many of these newly tested substances were active

TABLE II. Antitubercular Activities in Mice of Selected Thioureas.

Compound*	R	R_1	Approx. MTD (%)	% conc. in diet	T50	% survival	Infecting organism
Su 1495/E	H–	–H	.2	.1	19	10	H37Rv
1380	CH_3CH_2O–	$-OCH_2CH_3$	5	.1	>35	80	"
				.05	32.8	50	"
1515	$CH_3CH_2CH_2CH_2O$–	$-OCH_2CH_2CH_2CH_3$	5	.025	>35	60	"
				.01	30	50	"
1618	"	$-OCH_2CH_3$	4	.05	>35	100	"
				.025	25	20	"
1795	CH_3CH_2O–	$-OCH_2CH(CH_3)_2$	5	.05	>35	70	"
				.025	20	0	"
1814	$CH_3CH_2CH_2CH_2$–	$-CH_2CH_2CH_2CH_3$	>.5	.05	>35	100	"
1607	H_2N–	$-NH_2$	.05	.05	19.2	0	Ravenel
1566	$(CH_3)_2N$–	$-N(CH_3)_2$	.1	.1	21.5	0	"
1906	$CH_3CH_2CH_2CH_2O$–	"	.1	.025	>35	90	H37Rv
				.01	31	40	"
2211	$CH_3CH_2CH_2CH_2$–	$-OCH_2CH_2N(CH_2CH_3)_2$	>.1	.1	>35	90	"
				.05	>35	60	"

* The compounds were prepared by Drs. C. Huebner, J. Marsh, D. C. Schroeder and C. R. Scholz of our Chemical Research Division(2).

TABLE III. Effect of Delayed Therapy with Su 1795 on Mouse Tuberculosis.

Conc. of Su 1795 in diet, %	Treatment started	No. mice used	T50 (days)	% survivors
.5	At time of infection	10	>37	90
	3 days post-infection	10	>37	100
	6 " " "	9	>37	90
	9 " " "	10	>37	90
	12 " " "	10	>37	90
	15 " " "	9	>37	70
	18 " " "	10	19.5	30
.1	At time of infection	9	>37	80
	3 days post-infection	10	>37	90
	6 " " "	9	>37	100
	9 " " "	10	34	50
	12 " " "	10	24	40
	15 " " "	10	17	10
	18 " " "	8	17	0
Untreated controls	—	19	20.5	0

in vitro against the pathogenic mycobacteria and several species of actinomyces and fungi, but exhibited only insignificant activities against staphylococci, *E. coli, Shigella paradysenteriae,* Salmonella and other common bacteria, as was the case with the substances reported in the earlier study. However, this parallelism between anti-mycobacterial and antifungal action is not evident in all instances, and such irregularities are not unexpected. Substances commonly characterized as antibacterial, antifungal or as antiparasitical agents do not necessarily act upon all bacteria, fungi or parasites respectively but as a rule only affect certain species or more often, sub-species. It is probable that biochemical specificities, differences in permeability of the cell walls and many other factors play very important contributing roles. We feel that the observations reported in this paper support the theory that the botanical relationship between the mycobacteria and fungi is based not only upon superficial morphological characteristics but rather on profound biochemical re-

TABLE IV. Effect of Limited Therapy with Su 1795 on Mouse Tuberculosis.

Conc. of Su 1795 in diet, %	Total time of treatment, days	No. mice used	T50	% survivors
.1	3	10	27.5	30
	6	8	>44	70
	9	9	>44	80
	12	10	>44	100
	15	8	>44	80
.05	3	10	23.5	30
	6	10	33	50
	9	10	31	30
	12	9	>44	80
	15	10	>44	80
Untreated controls	—	19	29.0	10

lationships. As evident by this study, this theory has proven to be useful as a rational approach in the search for new antitubercular agents.

Certain disubstituted thioureas exhibited strong antitubercular activity in mice and guinea pigs. A number of compounds were highly active *in vitro* but inactive *in vivo*, while others had very low *in vitro* activity but considerable *in vivo* activity, suggesting that these latter compounds undergo a metabolic transformation into more active products. From these data it is obvious that within this class of compounds little relationship exists between *in vivo* and *in vitro* tuberculostatic activities.

The activities of certain substituted thioureas such as Su 1795 and Su 1906 in guinea pig experiments exceeded those of PAS or streptomycin, and approached that of Isoniazid.

Preliminary studies thus far have indicated that *in vitro* resistance develops slowly and that apparently no resistant forms of the tubercle bacillus arise in the course of animal

TABLE V. Summary of Mean Gross Organ Involvement in Guinea Pigs Treated with Four Thiourea Derivatives.

Compound	Approx.* MTD, %	% conc. in diet	No. of animals	Mean organ involvement: Spleen	Lungs	Liver	Site of inoculation	Total organ involvement
Su 1380	2.0	.05	12	5.8	5.0	6.7	5.8	23.3
		.2	13	4.6	2.3	5.4	1.5	13.8
Su 1795	1.0	.05	14	7.2	8.6	7.9	1.4	25.1
		.2	12	5.8	2.5	5.8	.8	14.9
Su 1814	>.2	.05	13	13.1	13.1	13.5	6.9	46.6
		.2	14	13.6	13.6	10.7	6.4	44.3
Su 1906	.1	.05	10	2.0	4.0	6.0	4.0	16.0
		.1	12	4.2	4.2	6.7	2.5	17.6
Isoniazid	.1	.05	13	3.1	3.1	3.1	2.3	11.6
		.1	13	1.5	1.5	2.3	.8	6.1
Tibione	.1	.05	12	9.2	10.8	12.9	5.0	37.9
		.1	12	2.5	7.5	9.2	5.0	24.2
Untreated controls	—	—	13	23.7	25.3	24.2	10.0	83.2
Pretreatment controls†		—	5	21.0	10.0	13.0	10.0	54.0

* The MTD is based on a 60-day feeding period. Values indicate percentage of drug in diet.
† These animals sacrificed on 21st day post-infection to ascertain degree of tuberculosis involvement at commencement of therapy.

TABLE VI. Influence of Delayed Therapy with Su 1795.

Compound	% conc. in diet	No. animals used	Avg values assigned to: Spleen	Lungs	Liver	Site of inoc.	Total score
Su 1795	.1	17	1.8	6.5	8.8	1.2	18.3
Isoniazid	.1	18	2.8	5.0	6.1	1.1	15.0
Untreated controls	—	17	27.4	21.2	24.1	10.0	82.7
Pretreatment controls	—	5	35.0	22.0	24.0	10.0	91.0

FIG. 1. Guinea pig experiment. Delayed therapy with Su 1795.

experiments. Cross-resistance studies have shown that infection of mice with a streptomycin-resistant strain of $H_{37}Rv$ yielded to the action of the thiocarbanilides, and Rimifon-resistant strains retained their sensitivity to the thiourea derivatives. The favorable results obtained in the delayed and limited therapy experiments together with the high therapeutic index suggest that certain of these compounds warrant consideration as therapeutic agents in the treatment of tuberculosis.

The mode of action of our compounds is still

unknown. With the collaboration of Dr. F. Kull and Miss M. Grimm of our laboratories, we have examined their influence upon various enzyme systems and found that their antituberculosis action is not based upon an antiphenoloxidase activity so pronounced with thiourea and certain monosubstituted thioureas nor due to an effect upon cytochrome C. A very high *in vitro* activity against different strains of torula, which contain as do the mycobacteria, a high percentage of lipids, may indicate a possible pathway for the tuberculostatic activity of these thioureas.

Summary. 1. Over 350 thioureas have been prepared in our laboratories and tested for antitubercular activity. 2. A number of disubstituted thioureas have demonstrated considerable protective and therapeutic activities in tuberculous mice and guinea pigs. 3. Little correlation existed between the *in vitro* and *in vivo* tuberculostatic action. 4. Many compounds possessed strong antifungal as well as antimycobacterial properties.

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Determination of Specific Gravity of Intact Animals by Helium Displacement; Comparison with Water Displacement.* (20242)

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Interest in the concept that the body is composed of a lean tissue mass of constant composition upon which is superimposed a variable amount of fat was stimulated by the work of Behnke(1). In normal men(2) and animals(3), the weight of fat-free tissue in the body as a whole or in individual tissues(4) is more closely correlated with the water and electrolyte content than is the weight of the total body or tissue. Since fat is relatively inert metabolically, the lean body mass may also be a more valuable referent for total metabolism than the surface area(5). Since the specific gravity of fat is about 0.92 and the specific gravity of the fat-free body is about 1.100(2), the proportion of body weight which is lean tissue and, conversely, the proportion which is fat, may be calculated from the whole body specific gravity. In man, specific gravity may be determined by underwater weighing, if correction is applied for the residual air in the lungs. This procedure is difficult to perform because it requires considerable training and cooperation on the part of the subject, and it is not feasible in incapacitated individuals. In intact animals this procedure is obviously impractical, and it is necessary to sacrifice and eviscerate animals in order to find their weight under water.

The method herein reported permits rapid determinations of specific gravity in intact, unanesthetized animals. Body volume is measured by gas displacement. Weight (g) divided by volume (cc) gives specific gravity, and no correction for residual air is needed.

Methods. Principle. A measured quantity of helium is added to a chamber containing the animal. After complete mixing of the gas with the air in the chamber and in the animal's lungs, a sample is obtained for analysis. Then, by the dilution principle: Volume of empty chamber $-\frac{\text{Vol. of gas added}}{\text{Final conc. of added gas}}$ = vol. of animal. Helium was selected as the referent

* The opinions expressed herein are those of the authors and should not be construed as necessarily representing the opinion of the naval service.

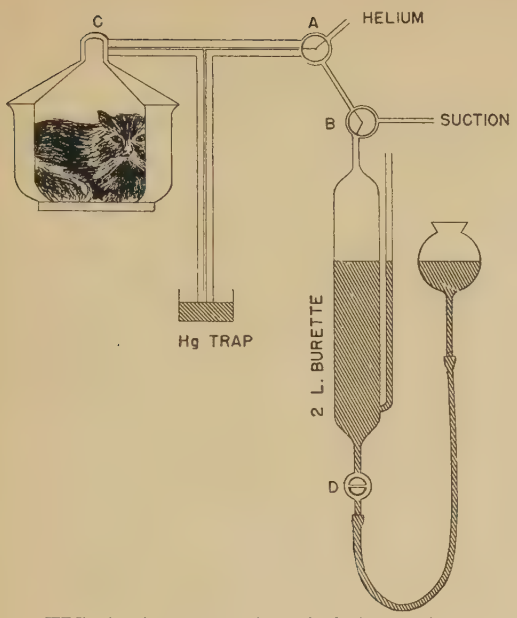

FIG. 1. Apparatus for admitting helium.

gas because it is inert, diffuses rapidly, and is the least soluble of the gases. Instead of enlarging the chamber in order to admit the helium at atmospheric pressure, it proved more convenient to evacuate the chamber partially and then add helium until atmospheric pressure was restored. *Apparatus* (Fig. 1). A 2-liter calibrated gas burette was filled with helium under slight positive pressure through stopcocks A and B. The pressure was reduced to ambient by releasing helium through the mercury trap, and the level of mercury in the burette recorded. (In view of the low solubility of helium in water, the use of mercury instead of water to displace helium was probably an unnecessary precaution.) The animal was placed in the chamber (an ordinary desiccator jar of exactly 6-liter capacity), and a negative pressure of approximately 20 cm Hg (measured by the height of the mercury above the trap) was produced therein by evacuation through C, A and B. Helium was admitted to the chamber from the burette through stopcocks B, A and C, by carefully manipulating stopcocks B and D, in order to maintain the pressure within the burette close to atmospheric. Thus it was possible to prevent temperature change in the burette. When the pressure within the chamber was restored to ambient, as indicated by the fall of the mercury above the trap to the level in the trap, stopcock C was closed. This procedure required less than one minute. The burette was read again and the volume of helium added obtained by difference. After 3-4 minutes (see below) a sample was obtained from the chamber for analysis and the animal was removed. *Experimental animals.* Cats weighing 2 to 3 kg were used. Most of the fur was removed with clippers before the experiment in order to facilitate subsequent underwater weighing of the carcass; air trapped in the fur was otherwise difficult to remove. *Analysis of helium.* Helium was de-

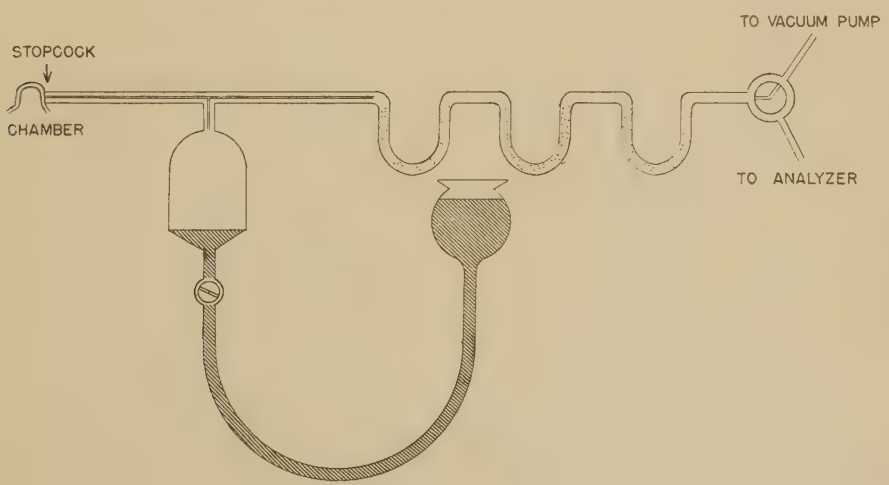

FIG. 2. Gas sampling apparatus.

TABLE I. Comparison of Specific Gravity Determinations by Gas Displacement and Water Displacement in 10 Cats.

Subject	Wt, g	Sp. gr. by He_2 Values	Mean	Sp. gr. by H_2O	Difference
H_2O	4000	.998 .991 .991	.993	.998*	—.005
Cat # 1	2605	1.080 1.070 1.070	1.073	1.050	+.023
2	2605	1.035 1.050 1.034	1.039	1.040	—.001
3	2519	1.023 1.051 1.027	1.033	1.048	—.015
4	2235	1.060 1.056	1.058	1.059	—.001
5	2552	1.083 1.074 1.073	1.077	1.071	+.006
6	2009	1.061 1.076	1.068	1.067	+.001
7	2060	1.087 1.083	1.085	1.077	+.008
8	2755	1.032 1.046	1.039	1.071	—.032
9	2512	1.057 1.067 1.039	1.054	1.051	+.003
10	2074	1.035 1.027	1.031	1.071	—.040
			Mean difference		.013
			Algebraic mean		—.005

* Density of water at room temperature.

termined in a Cambridge Analyzer, which utilizes the thermal conductivity of mixtures of helium and dry air to give per cent helium concentration. The gas sample was taken into an evacuated system (Fig. 2), passed through a drying agent and through the analyzer at a controlled rate of flow. *Determination of specific gravity by water displacement.* After sacrificing the animals and removing their lungs, the weight of the carcass suspended in water was determined. Care was taken to remove all air in the respiratory passages and that trapped in the remaining fur. The specific gravity of the lung-free carcass was calculated as:

$$\text{S.G.} = \frac{\text{Wt in air}}{\text{Wt in air—wt in water}}$$

Results. The results of the specific gravity determinations by helium displacement and by water displacement are shown in Table I. The arithmetic mean of differences between results obtained by the two methods was 1.3%. The validity of this procedure for measuring volume is indicated by the results obtained with a known quantity of water, also shown in Table I.

Discussion. Possible sources of error in gas displacement method. 1. Temperature effects: Since the 2 measurements, volume of gas added and helium concentration, are made at sites where the temperature and pressure are equal to ambient temperature and pressure, heating or cooling within the chamber does not affect the results, even though it alters the pressure. 2. Absorption of helium by the body: The solubility of helium in body water is approximately 0.0087 cc/cc(1), and in body fat approximately 0.0148 cc/cc(1). Complete equilibration of body water and fat with a helium atmosphere requires several hours(1). Consequently, the amount of helium removed from the atmosphere surrounding the animal during the time required for mixing is a negligible proportion of the total helium present, and will not affect the results; simple calculations show that even when complete equilibration is obtained the error is small. 3. Incomplete mixing: During normal respiration, equilibration of a foreign gas with alveolar air is 98% complete within 4 minutes(6). The reduction in pressure and restoration of atmospheric pressure by helium addition ensures that all gas spaces in or about the animal which are in communication with the atmosphere will be penetrated by helium, although equilibration with poorly ventilated recesses such as the paranasal sinuses may be incomplete. Mixing within the chamber was aided by the movements of the animals. That mixing was essentially complete was indicated by the fact that leaving a cat within the chamber for 3, 5 or 7 minutes gave the same result. Gas enclosed in spaces such as the stomach and intestines, will not be penetrated by helium. Consequently the results obtained by both the helium dilution and water weighing methods differ from the true body specific gravity by a small and variable amount.

Evaluation of gas displacement method. The results presented show that the volume of intact animals may be determined with fair

precision and accuracy by utilizing the gas dilution principle presented above. By varying the size of the chamber and the gas burette, it should be possible to perform repeated specific gravity determinations on intact animals of most species by this method. Knowledge of the empirical constants relating specific gravity to body fat and body water content should thus permit these latter quantities to be determined with ease and moderate precision.

Summary. A method of measuring specific gravity, and thereby estimating total body water and fat content in intact animals is presented. The dilution volume of helium, in a closed chamber of known capacity containing the animal, is measured, and subtracted from the volume of the empty chamber. This gives the volume of the animal. Weight/volume gives specific gravity. No correction for residual air is necessary. The results on 10 cats by this method corresponded closely to results obtained by underwater weighing of each carcass.

NOTE—Since this work was completed, an abstract has appeared(7) indicating that a helium dilution procedure has recently been applied to body volume measurement in man.

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Gonadotropins of the Pituitary Gland and Urine of the Adult Human Male. (20243)

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The repair of the gonadal atrophy occurring in hypophysectomized male and female rats provides a specific method for the characterization and assay of pituitary gonadotropins. Considerable information on the gonadotropins of the pituitary gland of the hog and the sheep has been derived by use of this method(1,2). However, only few definitive studies of the nature and amounts of gonadotropins of the pituitary gland of the human have been made, and much of what is known has been inferred from investigations on the gonadotropins of human urine(3-8). The purpose of this paper is to present comparative data on the gonadotropins of both the pituitary gland and the urine of the adult human male.

I. Materials and methods. a) Animals. Male and female Wistar rats (30 to 45 days old) were hypophysectomized by the parapharyngeal route. Postoperatively the animals received a solution containing 5% glucose and 0.9% sodium chloride as their drinking water and were allowed Friskie meal *ad libitum.* Beginning on the sixth postoperative day and extending for 4 days, each test animal received twice daily subcutaneous injections (1 cc) of the material to be tested. Groups of uninjected hypophysectomized and intact animals were included in each experiment. On the tenth postoperative day all the rats were killed by ether asphyxiation. A fairly complete gross and microscopic examination of the endocrine glands and other related organs was made. *b) Pituitary tissue.* The pituitary glands of 3 adult human males were obtained within 3 to 4 hours following sudden death.* None of the 3 had had any obvious endocrinopathy and in no case was there any clinical evidence of impairment of the pituitary-gonad system. The ages of the patients were 38, 55 and 61 years. The anterior lobe of each hypophysis was dissected free from the remainder of the

TABLE I. Effect of Human Pituitary Homogenate on Hypophysectomized Female Rats.

Animals			Ovaries		Uterus		Ovarian histology			
Condition	No.	Dose, mg	mg	mg/g	mg	mg/g	F.D.	C.L.	I.T.	V.O.
Hyp.	6	0	10.6	.13	36.6	.44	0	0	D	0
Intact	5	0	28.8	.25	159.3	1.39	+++	+	R	+
Hyp.	4	1	10.7	.10	75.4	.70	0	0	D	0
Hyp.	5	3	10.2	.13	29.7	.38	+	0	R	0
Hyp.	5	10	27.4	.40	136.3	2.01	++++	0	L	0
Hyp.	4	30	111.7	1.03	190.0	1.75	++++	+	L	+

Abbreviations: Hyp. = hypophysectomized; mg/g = organ wt/g body wt; D = deficient; R = repaired; I.T. = interstitial tissue; F.D. = follicular development; C.L. = corpora lutea; 0 = absent; + = present; L = luteinized; V.O. = vaginal opening.

gland, weighed, and frozen. The combined wet weight of the 3 anterior lobes was 1,051.4 mg. The glands were stored at –3°C for approximately 5 weeks. Immediately prior to injection, the 3 glands were homogenized, pooled, and suspended in 1% solution of sodium chloride. Groups of male and female hypophysectomized rats received individual total doses of pituitary homogenate equivalent to 1, 3, 10 and 30 mg of pituitary tissue (wet wt). *c) Urine.* Large volumes of urine were obtained from a group of healthy adult males. The pooled urine was worked up according to the method of Bradbury and associates(3). Groups of hypophysectomized male and female rats received a total dose per animal of urinary concentrate equivalent to 1,200 cc of pooled urine. This dose was selected because it was the minimal amount which produced a maximal increase in the uterine weight in the intact immature female rat. Each animal receiving the urinary concentrate was also given each day 0.5 mg of cortisone subcutaneously. In preliminary experiments cortisone therapy had substantially decreased the mortality rate due to the toxicity of large doses of urinary concentrate.†

II. Results. a) Pituitary homogenate in female animals (Table I). The presence of FSH is indicated by follicular stimulation in hypophysectomized rats. There was no effect at a dose of 1 mg. At 3 mg, follicular stimulation was produced without macroscopic enlargement of the ovaries. Ten milligrams of pituitary homogenate increased the weight of the ovaries to about 3 times that of the hypophysectomized controls, an increase associated with the presence of large cystic follicles. With 30 mg, the ovaries were 8- to 10-fold heavier than those of control rats, and contained many recent, completely luteinized follicles.

The *secretion of estrogen* is due to the synergistic action of FSH with small amounts of LH(10). Estrogen secretion, as judged from uterine weights, occurred at doses greater than 3 mg and increased with increasing dosage. The most copious estrogen secretion occurred at a dose of 30 mg since in addition to maximal uterine stimulation, vaginal opening also occurred.

The presence of LH is indicated by the repair of the ovarian interstitial "deficiency" cells(10-15). At 1 mg, the deficiency cells were unaltered. At 3 mg, repair was evident and with 10 mg, there was marked luteinization of the interstitial tissue. Complete luteinization of the granulosa cells occurred only in the 30 mg dose. At this dose, ovulation was presumed to have occurred, for no ova were

* This represented the earliest possible time following death that the pituitary tissue could be obtained. The glands appeared fresh. While some loss of gonadotropin may have occurred within the first few hours following pronouncement of death, we assume that the comparability of the results in this and subsequent papers is affected equally by this factor. In any case, by necessity, all of the studies on human pituitaries were done on glands obtained within three or four hours after death.

† Cortisone therapy did not impair the validity of the test, since (a) it did not influence the characteristic atrophy of the gonads and accessories following hypophysectomy, (b) it had no androgenic action on the accessories and (c) although it inhibits the growth of the accessories under androgenic stimulation(9), this effect is quantitative rather than qualitative and would make the positive results reported later even more significant.

TABLE II. Effect of Human Pituitary Homogenate on Hypophysectomized Male Rats.

Animals			Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Dose, mg	mg	mg/g	mg	mg/g	mg	mg/g	M.T.D.	S.A.	E.St.	L.St.	I.T.
Hyp.	8	0	5.3	.09	6.0	.11	28.4	.50	.09	+	0	0	U
Intact	5	0	37.8	.41	22.0	.24	87.0	.94	.16	++++	+	+	Di.
Hyp.	3	1	20.9	.42	13.3	.26	57.0	1.13	.09	++	0	0	U
Hyp.	4	3	25.2	.35	29.0	.40	70.0	.98	.11	+++	0	0	Di.
Hyp.	5	10	24.2	.40	22.2	.36	72.8	1.19	.13	+++	+	0	Di.
Hyp.	5	30	27.2	.38	22.9	.32	83.2	1.17	.11	+++	+	0	Di.

Abbreviations: M.T.D. = mean tubular diameter (mm); S.A. = spermatocytic proliferation; E.St. = early spermatid; L.St. = late spermatid; I.T. = interstitial tissue; U = undifferentiated; Di = differentiated; others, see Table I.

TABLE III. Effect of Urinary Concentrate on Hypophysectomized Female Rats.

Animals				Ovaries		Uterus		Ovarian histology			
Condition	No.	Cortisone	Concentrate	mg	mg/g	mg	mg/g	F.D.	C.L.	I.T.	V.O.
Hyp.	2	0	0	9.8	.15	19.5	.30	0	0	D	0
Hyp.	4	+	0	7.9	.15	14.7	.28	0	0	D	0
Hyp.	11	+	+	20.3	.36	68.6	1.23	++++	0	D	+
Intact	5	0	0	21.6	.24	73.4	.81	+++	+	R	+

Abbreviations: See Table I.

observed within the luteinized follicles.

Survey of the data showed that distinct qualitative differences in response were obtained over the dose intervals, which were purposely widely separated to minimize overlap. At a dose of 3 mg, the first distinctive indication for the presence of FSH was seen. This response was designated as a unit response; therefore, 1 unit of FSH was present in 3 mg pituitary homogenate. At 3 mg also the first distinctive response in the interstitial tissue (repair of deficiency cells) occurred. This response was therefore designated as a unit response to LH; therefore 1 unit of LH was present in 3 mg homogenate. The average weight of the pituitary was about 300 mg; therefore, a total of 100 units of FSH and 100 units of LH, as defined, were present per pituitary. It is plain that this criterion of FSH activity is made in the presence of LH, and of LH in the presence of FSH, a situation which will be discussed and clarified later.

b) Pituitary homogenate in male animals (Table II). In the hypophysectomized male rat the testicular interstitial spaces are almost totally obliterated by closely packed seminiferous tubules(11-16). The cells in these spaces cannot be readily identified as Leydig cells, but resemble fibroblasts or capillary endothelial cells. LH activity is defined by the ability of a preparation to induce differentiation of these cells or to induce secretory activity of these cells as manifested by androgenic stimulation of the prostate and seminal vesicles(15). The first direct *evidence of LH* activity occurred at a dose of 3 mg. The interstitial spaces were better delineated, being apparently distended by fluid or some other amorphous intercellular material. Leydig cells were easily identified. With 10 and 30 mg, an epithelioid cell pattern of the Leydig cells was produced. Indirect evidence of LH activity was present at all dosages studied, as judged from the weight and from histologic examination of the accessory glands.

The repair of tubular atrophy and reappearance of spermatogenic activity denotes the presence of FSH(12,16). The first definite response of the tubular apparatus as measured by testicular weight and, more especially, by tubular diameter occurred at 3 mg. There was an increase in spermatogenic activity over the entire dose range, with greater mitotic activity occurring at higher dosages. At no dosage, however, did the mitotic activity of the tubules of the hypophysectomized rats receiving pituitary homogenate equal that of intact control rats.

The same assignment of units described previously was made. The smallest dose of

homogenate necessary to repair the Leydig cells was 3 mg. This was designated as a unit response for LH. The smallest dose causing definitive tubular stimulation was also 3 mg. This was designated as a unit response for FSH. Therefore, as measured in the hypophysectomized male rat, there were roughly 100 units of FSH and 100 units of LH in the pituitary gland of the adult male.

c) Urine in female animals (Table III). The presence of LH was shown by the copious secretion of estrogen, resulting in uterine enlargement and patent vaginae. However, since there was no repair of the interstitial tissue, considerably less than 1 unit of LH was present. The *presence of FSH* was shown by the increased ovarian weight and the marked follicular stimulation. The follicles were large cystic structures without signs of luteinization. The FSH response was about equal to that brought about by a dose of between 3 and 10 mg (perhaps 6 mg would be an interpolated value) of pituitary homogenate.

d) Urine in male animals (Table IV). There was no evidence of LH activity as revealed by histologic examination of the interstitial cells. The weight of the ventral prostate was not appreciably increased but microscopic examination of the ventral prostate showed a moderate increase in the height of the acinar epithelium and dilatation of the acini with secretion. This slight androgenic effect indicated the presence of traces of LH, considerably less than 1 unit of LH. The *testicular weight* was increased to 250% of the hypophysectomized control. Spermatogenesis had progressed universally to the late spermatid stage. The increase in testicular weight and especially in mean tubular diameter was roughly equivalent to that produced by 3 to 10 mg (perhaps 6 mg) of pituitary homogenate.

III. Discussion. Henderson and Rowlands (17) and Chance and associates (18) have shown that the human pituitary is an exceedingly rich source of gonadotropins as compared to the hypophyses of other mammals such as the horse, the pig, and the hog. That it contains FSH and LH has been shown by Noble and associates (19) who obtained luteinization in the ovary of the hypophysectomized female

TABLE IV. Effect of Urinary Concentrate on Hypophysectomized Male Rats.

Animals				Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Cortisone	Concentrate	mg	mg/g	mg	mg/g	mg	mg/g	M.T.D.	S.A.	E.St.	L.St.	Sz.	I.T.
Hyp.	4	0	0	8.2	.15	12.0	.21	43.0	.77	.10	+	0	0	0	U
Hyp.	3	+	0	8.9	.16	9.9	.17	32.7	.57	.11	+	0	0	0	U
Hyp.	9	+	+	9.9	.18	10.9	.20	51.2	.91	.12	++	+	+	0	U
Intact	5	0	0	70.4	.60	53.6	.45	164.6	1.39	.21	+++	++	++	+	Di.

Abbreviations: See Table II. Sz. = spermatozoa.

rat with acetone-dried human pituitary. Bates and Schooley(20) concluded that the human pituitary contained more LH than that of the horse, the pig, or the sheep, but Witschi and Riley(21) and Witschi(22) reported that only traces of LH were present in the human pituitary.

It is difficult to compare these reports with one another and with the present data. Perhaps one difficulty is that different test animals were employed. The choice of units for FSH and LH may present another difficulty. For example, it would be entirely possible to choose an insensitive unit response for FSH activity, and a very sensitive unit response for LH. Under such circumstances, one could say that numerically, a great deal more LH than FSH was present. The reverse situation could also obtain. "Units" of FSH and LH are at present meaningless, since neither FSH nor LH is available in acceptable form with regard to either biological or chemical purity. Since biological purity is not assured, the effect of LH on the assay for FSH and vice versa in unfractionated pituitary tissue is largely unknown. Consequently, no absolute connotation is intended for our assay of 100 units of FSH per adult male pituitary. Rather this assay for FSH is defined as one carried out in the presence of, and perhaps excess of LH. The same consideration applied to the LH assay. Since chemical purity is not a characteristic property of existing gonadotropic preparations, and since no biologically pure but chemically impure reference standard preparations of LH and FSH are available, there can be no assay in terms of absolute or relative weights of the 2 hormones. It is impossible therefore to compare these 2 hormones in terms of the weights of each present in the pituitary, even if one were to dismiss the effects of synergism and interaction of these hormones on one another in the assay.

In order to circumnavigate partly the difficulties inherent in the present unsatisfactory situation, and to make some progress despite it, the following criteria were established: 1) Qualitative responses generally accepted as indicating the activity of FSH and LH were chosen as end points because of their distinctness and the ease with which they could be determined. 2) Doses of homogenate were chosen purposely separated by wide intervals (300%) so as to minimize or eliminate intragroup variation among animals, and to minimize minor variance in response due to differences in size and age of animals and to spontaneous statistical error. 3) Both male and female test animals were employed; in effect, 2 end points for FSH and 2 for LH were used. 4) The end points for FSH and LH were chosen because both appeared at the same dose level of homogenate in both male and female rats. By definition, the ratio of FSH and LH was made unity, so that gross departures from this ratio, as a standard for gonadotropin of the human pituitary, could be readily determined. 5) By determination of the amount of substance necessary to induce these end points, a rough calculation of the total gonadotropin present could be made. That this is a rough approximation and is not intended as a final meticulous assay is obvious from the use of a dose series separated by a log interval of 0.477, and by the existence of a factor of unknown quantitative significance, the factor of synergism.

Within the foregoing restrictions, the data show that about 100 units of FSH and 100 units of LH are present in the pituitary gland of the adult male. Comparative reports on similar material from females, children and infants will be made. At present, it is interesting to compare the gonadotropic content of the pituitary and urine of the adult man. In 1,200 g of pituitary tissue, some 400,000 units of FSH and LH are present (1,200 g/3 mg). In 1,200 cc of urine, perhaps 2 units of FSH and much less than 1 unit of LH were found.

These results have some bearing on a point which has been disputed for many years. This dispute concerns the duality of gonadotropins, which appears to have been settled, in a chemical sense, in the affirmative. However, Cole(23) in a recent review has suggested that LH may not be released from the pituitary and that, in fact, FSH is the only circulating gonadotropin. Studies on the blood of human beings are so fragmentary that no knowledge of the nature of circulating gonadotropin is at hand. Consequently, investigations of urinary concentrate, prepared by precipitation with

tannic acid or alcohol, by ultrafiltration, or by absorption methods, have been made to study the nature of excreted gonadotropins. Were the circulating gonadotropin only FSH, then one would expect no similarity between the activity of urinary concentrates and that of human pituitary tissue. On the contrary most of these studies on urine, including our own, have shown that both gonadotropic hormones are present. Our data, furthermore, show that proportionately much less LH relative to FSH is present and support Cole's suggestion to the extent that the ratio of the two gonadotropins in urine is not the same as that in the pituitary. The reason for such a disparity is not clear although a number of possibilities come to mind: differential secretion from the pituitary, differing routes of metabolism or utilization, or selective excretion or retention by the kidneys. It is also possible that the methods used to prepare urinary concentrates are much more effective for recovery of FSH than for LH. Which of these or other possibilities is the basis for the disparity must await further investigation.

Summary. A method is described whereby the ratio of FSH and LH present in human pituitary tissue can be ascertained in terms of well-known qualitative end points in the gonads of hypophysectomized rats of both sexes. Such end points, representing unit responses of FSH and LH, were chosen because they could be readily determined, and because they occurred at the same dosage of pituitary homogenate. It was thus determined that pooled homogenate of the pituitary glands of 3 adult men contained 100 units of FSH and 100 units of LH per pituitary, a ratio of 1:1. By comparison on an equal weight basis with pituitary tissue, the urine of adult men contained only minute amounts of gonadotropins, but proportionately, there was much more FSH relative to LH, in a ratio of much greater than 2:1.

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The Inhibition of Organic Nitro-Reductase by Aureomycin in Cell-Free Extracts. (20244)

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Several reports have recently appeared on the effect of aureomycin on various enzymatic reactions in mammalian tissue. Loomis(1) observed that the antibiotic specifically depressed phosphorylation in normal mitochondria without, however, inhibiting respiration. Brady and Bain(2) reported that aureomycin similarly uncoupled oxidative phosphorylation in rat liver and brain. On the other hand, Van Meter and Oleson(3) found that aureomycin, in the absence of citrate, completely inhibited the respiration of whole rat liver homogenates within 10 minutes. In the presence of citrate, the rate of oxygen consumption did not start to decline until after 30 minutes, and an additional 30 minutes elapsed before the inhibition approached that of the citrate-free preparations.

In contrast to these effects of aureomycin on mammalian tissue, the antibiotic has hitherto not been found to inhibit cell-free enzyme systems derived from bacteria. Such an effect is, however, described in the present paper. Smith and Worrel(4) found that whole cell preparations of *E. coli* can reduce the nitro group of chloramphenicol to the corresponding arylamine. Egami, Ebata, and Sato(5) extracted a soluble enzyme from *Streptococcus hemolyticus* which mediated this reduction. In this laboratory cell-free extracts of *E. coli* were found to have a similar effect; and in both whole cell preparations and extracts, aureomycin inhibited the reduction to arylamine of the nitro groups of chloramphenicol and p-nitrobenzoic acid.

Methods. E. coli (E-26) was grown in a peptonized milk medium. Fifteen grams of Bacto-peptonized milk were autoclaved for 30 minutes in 900 ml of distilled water. 7.5 g of K_2HPO_4 were dissolved in 100 ml of tap water and the pH was brought to 7.2-7.4 by the addition of H_2SO_4. After autoclaving, the phosphate solution was added aseptically to the autoclaved peptonized milk solution and 0.1 ml of sterile tributyl citrate was added to the mixture. Twenty liter amounts of culture were grown overnight with continuous aeration at 37°C. The cells were harvested by Sharples centrifugation and washed once with cold distilled water. The wet paste of washed cells was stored at —10°C with no loss of activity for periods up to 4 weeks. Cell-free extracts were prepared by grinding 4 g wet weight of the washed cells with 8 g of Alumina A-301 (6). The paste was extracted with 20 ml of cold water and centrifuged in the cold at 27,000 x g for 30 minutes. This extract was stable for at least 4 weeks if stored at —10°. The complete system, containing (a) either 0.003 M chloramphenicol or p-nitrobenzoate, (b) 0.05 M phosphate or tris buffer at pH 7.0-7.5, (c) 0.005 M cysteine, (d) 10-100 μg aureomycin HCl/ml, and (e) 1.5 ml of cell-free extract in a total volume of 5 ml was incubated at 37°C for one hour. Arylamine was then determined by a modification of the Bratton-Marshall technic(4).

Results and discussion. Table I, Exp. 1, shows the effect of various concentrations of aureomycin-HCl on the reduction of 1 mg/ml chloramphenicol under aerobic conditions. Similar results were obtained when the reaction was run anaerobically. Even at concentrations of aureomycin as low as 10 μg/ml, the inhibition of the reduction was significant. In other experiments, the inhibition by 50 μg/ml was as large as 85%. Essentially similar results were obtained using p-nitrobenzoic acid as the reducible substrate.

Cysteine proved to be necessary for the reduction of the nitro compounds by the *E. coli* extract and no significant amount of diazotizable amine accumulated in its absence. Equivalent concentrations of pyruvate and

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TABLE I. Inhibition of Chloramphenicol Reductase by Aureomycin.*

Exp. No.	Aureomycin-HCl, μg/ml	Chloramphenicol, μg/ml	Optical density†	% inhibition
I	0	0	.015	—
	0	1000	.660	—
	10	"	.380	43
	20	"	.320	51
	30	"	.272	59
	50	"	.279	58
	100	"	.244	63
II	0	100	.437	—
	100	"	.177	60

* Experimental details cited in text.

† At 550 mμ in the Coleman Junior Spectrophotometer. At this wavelength, 4 μg of p-aminobenzoic acid had an optical density of 0.160.

succinate were 1/3 as active and glutathione was approximately 1/5 as active as cysteine; while malate, fumarate, and α-ketoglutarate were inactive. Aureomycin inhibited the reduction to the same extent in all 4 cases. In the absence of the extract, cysteine did not itself reduce the nitro compounds even at a final concentration of 0.05 M, 10 times that used in the present experiments. It may be noted that although the *E. coli* extract was found in manometric studies to catalyze the oxidation of cysteine, aureomycin in concentrations which inhibited reductase activity, had no effect on that cysteine oxidation.

In Exp. 1, Table I, the amount of arylamine formed represents approximately 2% of the total chloramphenicol (1000 μg/ml). In similar experiments with chloramphenicol at 100 μg/ml (Exp. 2, Table I), 10 μg of arylamine were formed in one hour and 18 μg in 2 hours, and the degree of inhibition by aureomycin was essentially the same as when 1000 μg/ml chloramphenicol was used.

Under the same conditions as those used in the experiments of Table I, terramycin had little effect on the nitro-reductase activity of *E. coli* extracts. This result is surprising in view of its recently reported isomorphism with aureomycin(7).

In view of the reports that aureomycin uncouples oxidative phosphorylation in mammalian tissue(1,2), the effect of 2,4-dinitrophenol on the reduction of the nitro groups of chloramphenicol and p-nitrobenzoic acid by the cell-free bacterial extract was investigated. The reduction of these compounds was inhibited to the same extent by dinitrophenol as by aureomycin. Studies are in progress on the possible similarity in the underlying mechanism of these two inhibitors, and on the role of phosphate in the system.

Summary. 1. Aureomycin-HCl in concentrations of 10-100 μg/ml inhibits the reduction to arylamine of the nitro groups of chloramphenicol and p-nitrobenzoic acid by cell-free extracts of *E. coli* (E-26). 2. Under similar conditions, terramycin has little effect on the reduction. 3. 2,4-dinitrophenol inhibits the reduction of the organic nitro compounds to the same extent as aureomycin. 4. Studies are in progress on the possible similarity in the underlying mechanism of inhibition by aureomycin and dinitrophenol.

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In vitro Incorporation of C^{14} Glycine and S^{35} Sulfate into Protein by Human Colon Mucosa.* (20245)

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(Introduced by E. S. West.)

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The incorporation of radioactive amino acids into protein and smaller peptides by *in vitro* technics has been reported from many laboratories, including our own(1-7). The bulk of the reports deals with liver tissues either sliced or fractionated into a variety of cell free systems. Winnick *et al.*(8) reported the incorporation of labeled glycine into protein of intestinal mucosa. The present report presents data concerned with the incorporation of radioactive glycine into the mucosa of resected human colon, into the insoluble protein and into a soluble fraction which has characteristics of a mucoprotein.

Materials and methods. Colon specimens were obtained from resections performed by the Surgical Service of Multnomah County Hospital. Pieces of normal bowel were used. The technic for handling the tissue after washing in 8-10 aliquots of buffer was the same as reported with liver biopsies(6) except that it was found necessary to dissect "strips" of mucosa from the colon tissue, since slicing was difficult. The "strips" were cut into small segments approximately 0.5 to 1.0 cm square, pooled, and then used for the incubation. Glycine-1-C^{14} (0.33 μc)† and 2 cc of a modified Krebs-Henseleit buffer‡ were added to 150-200 mg of wet weight of colon mucosa in each flask, which was then filled with 95% O_2-5% CO_2. The subsequent incubation, precipitation with boiling 1 M acetate, and isolation of protein for counting were the same as previously reported(6). In some experiments tissue was incubated with S^{35} labeled inorganic sulfate instead of glycine. In earlier experiments 1000 units of penicillin were added to each flask, and later aureomycin, 200 γ in 0.1 cc water, was substituted. The aureomycin solution was freshly made for each experiment. A mucoprotein fraction was obtained by a modification of the technic described by Glass and Boyd(9). Colon mucosa was homogenized, the protein precipitated with boiling 1 M acetate (pH 4.5), and 1.5 volumes of acetone were added to the supernatant. This solution was allowed to stand overnight at room temperature. The amorphous precipitate, after washing and drying with acetone, resembled a gastric mucoprotein described by Glass, contained 11.5% nitrogen and 0.95% sulfur by analysis, and gave a positive reaction to the uronic acid test described by Siplet, Komarov, and Shay(10). The 1 M acetate supernatants from incubated samples were dialyzed overnight against running tap water to remove unincorporated labeled glycine or sulfate. The mucoprotein was then precipitated with 1.5 volumes of acetone and prepared for counting. Tissue and labeled substrate were precipitated at zero time in each experiment as controls. Duplicate samples were run for each set of experimental conditions and the values were expressed as the means of duplicate pairs. Mucoprotein, however, was precipitated from the supernatants of duplicate pairs which had been pooled in order to obtain adequate amounts for handling. For the reprecipitation experiments, the dry protein was dissolved in 0.5 N sodium hydroxide to which 10-15 mg urea had been added. Protein was reprecipitated with 10% trichloracetic acid.

Results. Table I presents the effects of penicillin and aureomycin on incorporation. There was a consistent depression in the presence of penicillin and a more variable result with aureomycin. In order to evaluate the role

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† Glycine-1-C^{14}, 0.5 mc/millimole, purchased from Tracerlab, Boston, Mass.

‡ Composition previously described(6).

TABLE I. Effect of Penicillin and Aureomycin on Radioactive Glycine Uptake by Colon Mucosa.

Patient	Specific activity counts/min./mg — With penicillin, 1000 u	Without penicillin or aureomycin	With aureomycin, 200 γ
H.	822	1175	
O.	633	939	
S.	142	881	
R.	278	364	383
G.		568	326
B.		353	426
K.		457	666

of bacteria more definitively, slices of colon mucosa were compared to the same weights of tissue homogenized with the Potter glass homogenizer. This technic is known to destroy whole cells, and it is extremely unlikely that bacteria were destroyed. The homogenate was allowed to remain in the refrigerator overnight to insure destruction of the enzymes of the mucosa cells. The homogenate was incubated for 4 hours under experimental conditions and samples were then cultured in broth by the serial dilution technic. Table II demonstrates that incorporation into the mucosa was practically abolished by homogenizing. On the other hand bacteria were present in significant amounts, and a bacteriostatic effect of aureomycin was demonstrated.

In Table III are presented data which show no consistent effect as a result of omitting magnesium and calcium from the buffer. This is in contrast to the results previously obtained with liver slices(6). In the absence of citrate (patients K and B) the specific activities, although apparently higher, are probably of the same order of magnitude as with the complete buffer. Marked inhibition was observed in the presence of .005 M cyanide and in a nitrogen atmosphere. There was no apparent correlation between the colon site and the level of incorporation.

Data with respect to the incorporation of labeled glycine and labeled sulfate into both total protein and mucoprotein are presented in Table IV. Since the amount of S^{35} sulfate added varied, the specific activities cannot be compared with one another. In patients M and J the incorporation of labeled sulfate into the mucoprotein was obviously greater than in total protein. In contrast the incorporation of labeled glycine into the total protein was greater than into the mucoprotein in patients M, L and J. Solution of the colon mucosa protein in alkaline urea and reprecipitation with trichloracetic acid failed to remove any radioactivity when the labeling agent had been C^{14} glycine, whereas the sulfate label was entirely removed by the same procedure (Table V).

The figures presented are average values for duplicate pairs of observations for each set of experimental conditions except as noted. The variation of each observation from the mean of its pair of duplicates was less than 15% in 32 and less than 25% in 46 out of 58 pairs.

Discussion. There is considerable evidence indicating that the incorporation of amino acids into protein is an enzymatic process fundamentally requiring oxidative phosphorylation(11,12). The data presented do support the hypothesis that the incorporation of radioactive glycine into colon protein is probably enzymatic, especially since homogenizing the tissue abolished incorporation, and since both a nitrogen atmosphere and small concentrations of cyanide produced marked inhibition. The evidence rules out bacteria as being responsible for the incorporation. The percentage of labeled glycine incorporated is many times greater than the low levels of incorporation obtained by Brunish and Luck with their histone system(13). If the incorporation observed in our experiments were due to some artefact or adsorption rather than to a true enzymatic process, one would expect much higher levels of incorporation in the homogenized tissue (Table II) than in tissue with intact cells. Furthermore, solution with alkaline urea and reprecipitation with trichloracetic acid or treatment with mercaptoethanol has been shown to remove some radioactivity from labeled protein obtained from particulate systems and not from systems with intact cells. We were unable to alter the specific activity of our C^{14} glycine labeled protein by such treatment.

The marked variation in specific activities obtained from patient to patient cannot be readily explained. The same variation has been noted with liver tissue. The lack of sensitivity of the system to the presence or ab-

TABLE II. Radioactive Glycine Uptake and Bacterial Growth in Colon Mucosa Homogenate.

Patient	Specific activity: Slices with aureomycin	Specific activity: Homogenate With aureomycin	Specific activity: Homogenate Without aureomycin	Highest dilution showing growth: With aureomycin	Highest dilution showing growth: Without aureomycin
S.	279	3	3.	10	10^3
R.	444	3	12.	10^5	10^7

TABLE III.

Patient	Colon site	Specific activity: Complete buffer	No Ca	No Mg	No Ca or Mg	No citrate	N^2	CN^-
O.	Transverse	632	694	278	134			11
R.	Sigmoid	244	355	180	125			22
S.	"	279	278	225	210			17
M.	Splenic flexure	230‡*	581*	238*	358*			
L.	Rectosigmoid	124	106	127	610			
B.	"	177	723	172		248		
K.	Sigmoid	725				824		
G.	Rectosigmoid	326					23	
F.S.	"	142†					32†	43†
O.	"	634†					55†	
N.	Sigmoid	215*		147				
		65						
B.	Transverse	426						
A.S.	Rectosigmoid	364						
M.S.	Transverse	889						
H.	Rectosigmoid	822†						
Zero time mean ± S.E.		6.3 ± 1.3						

* No antibiotic present. All others contained 200 γ aureomycin.
† 1000 units penicillin added. No aureomycin.
‡ Single observation. All others are averages of duplicate pairs.

TABLE IV. Incorporation of Labeled Substrate into Protein and Mucoprotein of Colon Mucosa.

Patient	Specific activity: Total protein C^{14} glycine	Total protein S^{35} sulfate	Mucoprotein†‡ C^{14} glycine	Mucoprotein†‡ S^{35} sulfate
A.	364	155		
B.	410	177		
	353*	207*		
N.	65	47		
	215*	50*		
M.	230†	27	185	440
	580		427	
	358		270	
	238		200	
L.	124		40	
	610		265	
	127		61	
J.	240	100	140	2820

* Without aureomycin. All other exp., 200 γ aureomycin present.
† Single observation. All others are averages of duplicate pairs.
‡ In each exp. mucoprotein was isolated from supernatant from which total protein had been precipitated.

sence of added Ca^{++} or Mg^{++} in the buffer in contrast to the more easily demonstrable sensitivity to these ions in the liver might be explained by a more variable concentration of these ions in the colon. We have no data to substantiate or refute this possibility.

The incorporation of S^{35} sulfate into the protein and mucoprotein is a little more difficult to defend as a true enzymatic process, although such a process is certainly compatible with the data presented. Boström and

TABLE V. Reprecipitation of Labeled Colon Protein.

Patient	Label	Specific activity: Before solution	Specific activity: After reprecipitation
C.S.	C^{14} glycine	274	284
A.S.	C^{14} "	404	464
C.G.	C^{14} "	326	530
Pooled protein A	S^{35} sulfate	96	7
" B	S^{35} "	96	2

Mansson(14) have shown that cartilage slices will incorporate labeled sulfate into its mucoprotein, and that this process is inhibited by a nitrogen atmosphere. Dziewiatkowski(15) demonstrated that after giving S^{35} sulfate intravenously, intestinal mucosa was found to have significant radioactivity. We have obtained a protein with labeled inorganic sulfate which cannot be dialyzed away, yet is easily removed by solution and reprecipitation. This is compatible with the concept that the protein contained a coprecipitated mucoprotein which was easily removed by solution and reprecipitation. Winzler *et al.*(16) have demonstrated that serum mucoprotein is coprecipitated with serum protein.

The specific activity of the total crude protein, when labeled with C^{14} glycine, was consistently higher than that of the mucoprotein obtained from the same experimental conditions. The data are not sufficiently quantitative to draw any inferences with respect to precursor relationships. Since the mucoprotein contains only 12% nitrogen, the larger proportion of non-protein components in the mucoprotein might well account for the lower specific activities.

Summary. 1. The incorporation of carboxyl C^{14} labeled glycine into the protein of human colon mucosa has been studied *in vitro*. The incorporation was inhibited by nitrogen and cyanide. There was no consistent effect from omitting calcium and/or magnesium from the buffer. Penicillin depressed the incorporation; aureomycin produced no consistent results. Homogenized tissue failed to incorporate even though viable bacteria were present. 2. A mucoprotein was isolated from colon mucosa and both carboxyl C^{14} glycine and inorganic S^{35} sulfate were found to be incorporated into protein and mucoprotein. 3. Solution and reprecipitation of total crude protein removed the S^{35} label, but not the C^{14} label.

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